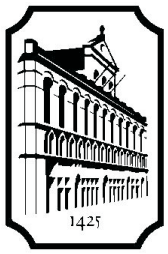
ACTA BIOMEDICA LOVANIENSIA 713
KU Leuven
Group Biomedical Sciences
Faculty of Medicine
Department of Cardiovascular Sciences
Anesthesiology and Algology

An MARTENS

NOVEL STRATEGIES TO INCREASE THE NUMBER AND QUALITY OF DONOR LUNGS FOR TRANSPLANTATION



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Thesis submitted in partial fulfillment of the requirements for the degree of «Doctor of Biomedical Sciences»

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“Success is a journey, not a destination”

Arthur Ashe

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DANKWOORD

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Be Thankful

Be thankful that you don't already have everything you desire,
If you did, what would there be to look forward to?
Be thankful when you don't know something,
For it gives you the opportunity to learn.
Be thankful for the difficult times,
During those times you grow.
Be thankful for your limitations,
Because they give you opportunities for improvement.
Be thankful for each new challenge,
Because it will build your strength and character.
Be thankful for your mistakes,
They will teach you valuable lessons.
Be thankful when you're tired and weary,
Because it means you've made a difference.

-Author Unknown-

ABBREVIATIONS

A2A	Adenosine 2 receptor agonist
ABP	Arterial blood pressure
ADR	Adrenaline
Akt	Protein kinase B
Alb	Albumine
Alk fosf	Alkaline phosphatase
ALT	Alanine aminotransferase
ANG	Angiopoietin
APAF	Apoptotic protease activating factor
Ar	Argon
ARDS	Acute respiratory distress syndrome
AST	Aspartate transaminase
ATP	Adenosine triphosphate
BAL	Bronchoalveolar lavage
BAX	Bcl-2-associated X protein
Bcl-2	B-cell lymphoma 2 protein
Bic	Bicarbonate
BID	BH3 interacting-domain death agonist
Bili	Total bilirubin
BOS	Bronchiolitis obliterans syndrome
Ca	Calcium
CCO	Continuous cardiac output
CD	Cluster of differentiation
CF	Cystic fibrosis
CFSE	Carboxyfluorescein sucinimidyl ester
CIT	Cold-ischemic time
Cl	Chloride
CLAD	Chronic allograft dysfunction
CMV	Cytomegalovirus
CO	Cardiac output
COMPL	Dynamic lung compliance
COPD	Chronic obstructive pulmonary disease

CRP	C-reactive protein
CT	Computed tomography
CVP	Central venous pressure
CVVH	Continuous veno-venous hemofiltration
DBD	Donation after brain death
DCD	Donation after circulatory death
DNA	Deoxyribonucleic acid
ECD	Extended-criteria donor
ECMO	Extracorporeal membrane oxygenation
EGF	Epidermal growth factor
ERK	Extracellular signal–regulated kinases
ET	Eurotransplant
$ETCO_2$	End-tidal carbon dioxide
EVLP	Ex-vivo lung perfusion
FEV_1	Forced expiratory volume exhaled in one second
FiO_2	Fractional inspired oxygen concentration
FVC	Forced vital capacity
GABA	Gamma-aminobutyric acid
Gamma GT	Gamma glutamyl transpeptidase
Gluc	Glucose
Hct	Hematocrit
HIF-1α	Hypoxia-inducible factor 1-alpha
HLA	Human leukocyte antigen
HR	Heart rate
I:E	Ratio of the duration of inspiration to the duration of expiration
ICU	Intensive care unit
IDO	Indoleamine-2,3-dioxygenase
IFN	Interferon
IL	Interleukin
iNO	Inhaled nitric oxide
INR	International normalized ratio (of the prothrombin time)
IQR	Interquartile range
IRI	Ischemia-reperfusion injury
ISHLT	International Society of Heart and Lung Transplantation

ISS	Injury severity score
IT	Intratracheal
IV	Intravenous
K	Potassium
KGF	Keratinocyte growth factor
LAP	Left atrial pressure
LDH	Lactate dehydrogenase
MAP	Mean arterial blood pressure
MAPC	Multipotent adult progenitor cell
MAPK	Mitogen-activated protein kinase
MCP-1	Macrophage chemoattractant protein-1
MHC	Major histocompatibility complex
MMP	Matrix metalloproteinases
MOD	Multi-organ donor
MP	Methylprednisolone
MSC	Mesenchymal stem cell
mTOR	Mammalian target of rapamycin
Na	Sodium
NADPH	Nicotinamide adenine dinucleotide phosphate
NC	Negative Control
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NIH	National Institute of Health
NMDA	N-methyl-D-aspartate
NORADR	Noradrenaline
NS	Not significant
OCS™	Organ Care System (Transmedics, Andover, USA)
Osm	Osmolality
PAF	Platelet-activating factor
PaO_2	Partial oxygen pressure
PAP	Pulmonary artery pressure
PC	Positive Control
PEEP	Positive end-expiratory pressure
PGD	Primary graft dysfunction
PGE_2	Prostaglandin-E_2

PI3K	Phosphatidylinositol-3-kinase
Ppeak	Peak pulmonary pressure
PT	Prothrombine time
PVR	Pulmonary vascular resistance
QI	Quartile interval
qPCR	Quantitative polymerase chain reaction
RBC	Red blood cells
RI	Hepatic artery resistive indices
RLL	Right lower lobe
ROS	Reactive oxygen species
RR	Respiratory rate
RUL	Right upper lobe
SCD	Standard-criteria donor
SSLTX	Sequential single lung transplantation
SvO_2	Mixed venous oxygen saturation
TCC	Total cell count
TGF-β	Transforming growth factor beta
THAM	Thromethamol
TIPS	Transjugular intrahepatic portosystemic shunt
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor alpha
TREK	TWIK-related potassium channel
TV	Tidal volume
VEGF	Vascular endothelial growth factor
VVB	Veno-venous bypass
W/D	Wet-to-dry weight ratio
WBC	White blood cells
WIT	Warm-ischemic time
Xe	Xenon

CHAPTER I

GENERAL INTRODUCTION

A) LUNG TRANSPLANTATION

Lung transplantation (LTx) is a life-saving surgical procedure in which diseased native lungs in patients suffering from end-stage pulmonary diseases are replaced by donor lungs. According to the ISHLT registry, the most common end-stage pulmonary diseases listed from 1995 up to 2014 where lung transplantation was indicated, were: 1) chronic obstructive lung disease (32%), 2) idiopathic pulmonary fibrosis (24%), 3) cystic fibrosis (16%), 4) alfa-1-antitrypsin deficiency (5%) and 5) idiopathic pulmonary arterial hypertension (3%). Since James Hardy performed the first lung transplant procedure in 1963 (1), the success of lung transplantation has grown enormously due to improved surgical techniques, perioperative management, immunosuppressive regimens, and strict long-term follow-up. Consequently, the overall survival for adult recipients is improving per era and the median survival of adults transplanted between 1990-2013 was 5.6 years (Figure I.1).

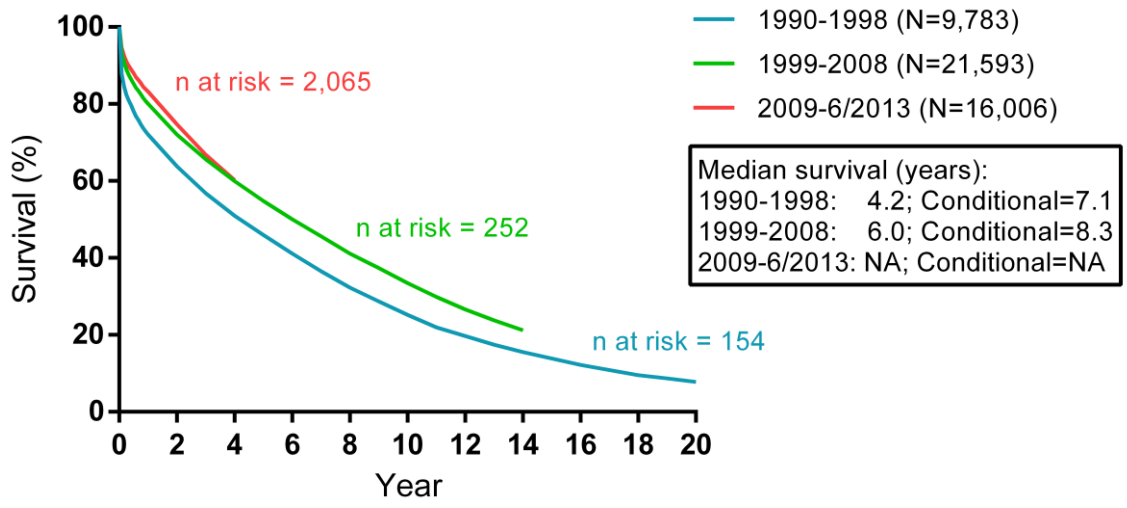


Figure I.1 Kaplan-Meier survival per era. Figure adapted from ISHLT Adult Lung Transplantation Statistics 2015. http://ishlt.org/registries/slides.asp?slides=heartLungRegistry

Nowadays, more than 4000 procedures are performed annually worldwide (2). At the University Hospitals Leuven, the lung transplant program initiated in 1991, has grown up to a mean annual transplant rate of 61 procedures over the past 5 years (2011-2015) (Figure I.2).

The worldwide growth in LTx is mostly due to an increase in sequential single lung transplantation (SSLTx), where both diseased lungs are transplanted (=bilateral lung transplantation). The number of single lung transplant (SLTx) procedures, where only one lung is transplanted, is less frequently used due to an inferior survival of the recipient compared to SSLTx (3).

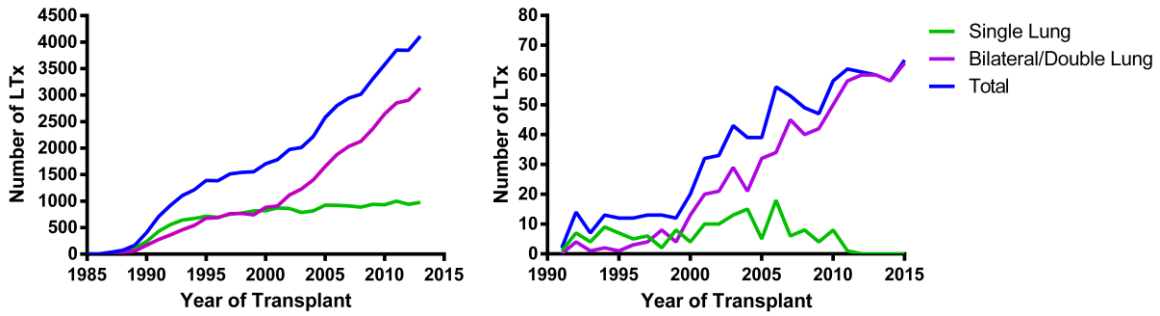


Figure I.2 –The annual number of single lung, bilateral/double lung and total lung transplant procedures registered at the International Society of Heart and Lung Transplantation (ISHLT) are depicted on the left. Lung transplant numbers from our University Hospitals Leuven are depicted on the right.

The success of LTx is critically dependent on the number and quality of donor lungs. This is currently one of the main challenges to maintain and improve the outcome for recipients and patients on the waiting list. Currently, the overall number of available donor lungs is still inadequate (2). This limits the further expansion of lung transplant programs and leads to a persistent 15 % mortality for patients on the waiting list (2,4). In addition, inferior quality of the transplanted donor lung can lead to primary graft dysfunction (PGD), which is a form of acute lung injury appearing shortly after lung transplantation. PGD can be considered as an important parameter of overall donor lung quality as it determines early outcome after LTx, but also acute and chronic rejection and even long-term survival might be linked to PGD (5,6).

B) DONOR ORGANS FOR LUNG TRANSPLANTATION

Solid organ transplantation is only possible if a suitable organ is donated. The main source for organ donors in lung transplantation, are recently deceased patients, referred to as cadaveric organ donors. Cadaveric organ donation is based on two types of donors: on the one hand, donors who die after brain death (DBD) and on the other hand donors who die after circulatory death (DCD). A person can become a cadaveric organ donor when he actively consented to be an organ donor (opting-in legislation), or when he did not register himself objecting to donate his organs (opting-out legislation).

Brain death in DBD is still a clinical diagnosis, based on the cessation of clinical functions of the brain that will not resume. It is determined by the absence of capacity for consciousness, centrally mediated motor responses, brainstem reflexes, and capacity to breathe (7). Donor organs remain perfused as the heart of the DBD is still beating at the time of organ procurement. However, the process of brain death is associated with a systemic inflammation and leads to an autonomic dysregulation (storm + depletion) that also damages the donor lung by detrimental effects on the cardiovascular function. In practice, the majority of lung transplants still results from these DBDs. That is, only 2-32% of all lung transplants results from DCD organ donation (8,9). And although the first performed lung transplant was from a DCD donor, the use of DCD donors to enlarge the donor pool has only been reintroduced in the early 90's (10,11).

A DCD donor, is a donor with a critical medical condition where further treatment is considered futile and the decision is made to proceed with end-of-life care. Most often, these patients suffered devastating and irreversible brain injury, but do not meet the formal criteria of brain death. Withdrawal of care in these patients will lead to circulatory arrest and death, whereafter organs can be procured for donation. Therefore, with DCD, there is a certain delay between circulatory arrest (cessation of organ perfusion) and the start of organ cooling (cold ischemia).

This interval, called the asystolic warm-ischemic time (WIT), will rapidly lead to cellular damage and compromise organ function and quality.

DCD donors were initially categorized (Table I.1) from I-IV according to the Maastricht classification (12). The Maastricht classification was later on modified and complemented with a fifth category in 2000 (13,14). Maastricht categories I and II (and V) are uncontrolled DCD donors (referring to the uncertainty of the exact moment of circulatory arrest), whereas class III and IV are controlled DCD donors (referring to the fact that the precise moment of circulatory arrest is documented). In the latter, treatment withdrawal in or near the operating room is followed by a stand-off period after cessation of circulation in the patient, followed by the declaration of death. After this controlled context of therapy withdrawal and circulatory arrest, the donor organs are retrieved. In contrast, uncontrolled DCD donors are found after circulatory arrest, and cardiopulmonary resuscitation is initiated in some of these patients until they are declared dead whereafter organ preservation measures can be started.

Table I.1 – Modified Maastricht Classification of donors who die after circulatory arrest (DCD) (13)

Category	Definition	Procurement
I	Brought in dead	Uncontrolled
II	Unsuccessful resuscitation	Uncontrolled
III	Awaiting cardiac arrest	Controlled
IV	Cardiac arrest after brain-stem death	Controlled
V	Cardiac arrest in a hospital inpatient	Uncontrolled

Currently, the lung retrieval rate (% of donors eligible for lung donation) of a multi-organ donor remains as low as 20 to 30% (2,15). In combination with the growth in lung transplant programs, this had led to a critical shortage in usable donor lungs (Figure I.3). Over the past 5 years, the discrepancy between the amount of available donor lungs and the number of patients listed is

shrinking (but still existent), due to efforts made by the entire transplant community. Unfortunately, there remains a high mortality rate on the waiting list. Between 2005 and 2013, 14.4% of patients listed for lung transplantation in the United States of America either died or were removed from the waiting list since they became too sick for a lung transplant procedure (4). In the same analysis, this percentage is as high as 22% for pediatric patients. Also within the Eurotransplant database, we report a persisting average mortality of 10% on the lung transplant waiting list between 2005 and 2015 (Eurotransplant database registry reports, Figure I.3). Therefore, the first major problem identified and addressed in this project will be:

Problem 1: A shortage of suitable donor organs leads to a persistent mortality for patients on the waiting list and limits further increase in transplant activity.

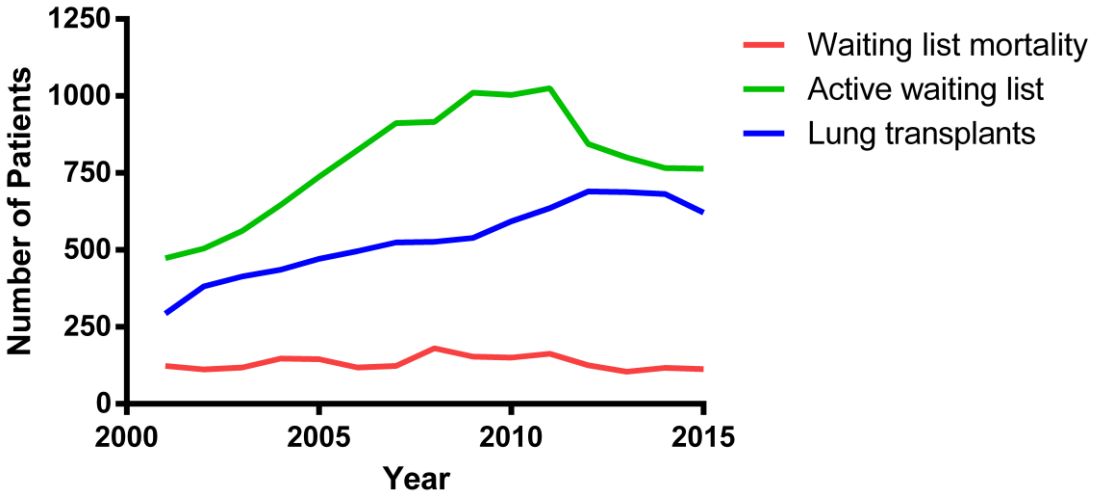


Figure I.3 – Eurotransplant data depicting patients active on the waiting list, total number of patients transplanted, and waitlist mortality within the Eurotransplant database (2).

For all types of organ donors, strict criteria of a "standard-criteria donor" (SCD) have been defined historically, to be considered as an ideal lung donor candidate (Table I.2) (16,17). However, to increase the amount of transplantable donor organs, these criteria have been liberalized to "extended-criteria donors" (ECD). These are lung donors not matching the strict criteria of a SCD.

In our center, ECD lungs account for 63% of donor lungs. Percentages range from 30-60% (18,19) in other centers, yet an extended criteria donor (ECD) is defined differently in numerous reports (18). Although the use of ECD lungs does not impair long-term clinical outcome in our center, a negative impact on early outcome (prevalence of severe primary graft dysfunction, length of stay in intensive care unit (ICU), duration mechanical ventilation) has been reported (20). A recent analysis of the UNOS registry did show a reduced one-year survival in patients receiving ECD lungs, with the lowest survival in patients with a lung allocation score of 70 or greater (21). However, several centers are reporting similar long-term outcome with ECD compared to SCD. Therefore, we start to understand that not all ECD donors should be considered as "marginal" (20,22,23). High-risk donor organs can be considered for transplantation, but should be allocated wisely to the most suitable recipient.

Table I.2 – Ideal lung donor criteria, adapted from Sundaresan et al. (17)

Ideal lung donor criteria
-Age < 55 yr
-Clear serial chest X-ray
-Normal gas exchange (PaO_2 > 300 mm Hg on FiO_2 = 1.0, PEEP 5 cm H_2O)
-Absence of chest trauma
-No evidence of aspiration or sepsis
-Absence of purulent secretions at bronchoscopy
-Absence of organisms on sputum gram stain
-No history of primary pulmonary disease or active pulmonary infection
-Tobacco history < 20 pack-years
-ABO compatibility
-No prior cardiopulmonary surgery
-Appropriate size match with prospective recipient

C) PRIMARY GRAFT DYSFUNCTION

Primary graft dysfunction (PGD) is a common complication immediately following transplantation of the lung graft, resulting in acute failure of its function. In the process of organ donation and lung transplantation, several harmful hits to the donor lung occur that may result in primary graft dysfunction (PGD). It already starts in the donor where prolonged mechanical ventilation, brain death or warm ischemia can trigger this injurious process. It continues during cold ischemic storage in preservation solution and peaks upon reperfusion inside the recipient's chest with possible further injurious insults to the transplanted lungs in the peri-operative phase. Before the establishment of the clinical definition of PGD by the ISHLT PGD working group in 2005 (24), it was often referred to as ischemia-reperfusion injury (IRI), early graft dysfunction, primary graft failure or re-implantation edema.

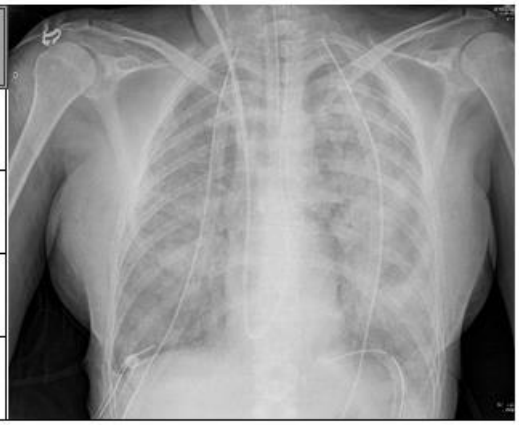
Clinical classification of primary graft dysfunction

PGD occurs per definition within the first 72 hours after lung transplantation and is characterized by severe hypoxemia, apparent lung edema with diffuse alveolar damage and radiographic evidence of diffuse pulmonary infiltration without other identifiable cause (mechanical, immunologic or infectious causes that can mimic or confound the diagnosis of PGD). Since 2005, it is categorized by the ISHLT PGD working group as grade 0 to 3 based on oxygenation and chest X-ray (Table I.3) and is scored at several time points after reperfusion (T0, T12, T24, T48, T72) (25). Severe (grade 3) PGD occurs in 10-30 % of cases (26).

Radiographic and histological findings are similar to acute respiratory distress syndrome (ARDS) (27,28). Initial epithelial denudation, in parallel with endothelial damage, leads to capillary leakage with interstitial edema that rapidly spreads to intra-alveolar edema which can be seen as diffuse parenchymal opacities on chest X-ray.

Table I.3 – Gradation of PGD

Grade	PaO_2/FiO_2	Chest X-ray infiltrates
0	>300	Absent
1	>300	Present
2	200-300	Present
3	<200	Present



PGD gradation is based on PaO_2/FiO_2 (PaO_2: partial oxygen pressure, FiO_2: fraction of inspired oxygen) and radiographic infiltrates that are consistent with pulmonary edema (right panel) (25)

Outcome

PGD results in impaired early outcome since it leads to prolonged length of mechanical ventilation, prolonged intensive care stay, prolonged hospital stay and even increased short-term mortality (29–31). Severe (grade 3) PGD at 48 and 72 hours is linked with an increased 90-day and 1-year mortality, with an absolute increase in mortality of 23% at 1 year (26). Interestingly, recent publications even show an impact on the later development of bronchiolitis obliterans syndrome (BOS), a phenotype of chronic allograft dysfunction (CLAD) (32,33), that limits long-term survival. PGD is associated with induced pro-inflammatory cytokines that can upregulate donor specific antigens to promote the development of donor allo-immunity (34). Increased levels of IL-6, IL-8 and TGF-β in bronchoalveolar lavage fluid (BAL) in patients with severe PGD (35–37) form an important mechanistic link between early post-transplant lung allograft injury and reported association with BOS.

Pathophysiology

The pathophysiology of primary graft dysfunction is the end-result of several injurious insults to the donor lung inside the donor, during preservation and upon reperfusion in the recipient. PGD results from a complex interplay between direct damage by ischemia and cold preservation, induction of cell death, production of reactive oxygen species (ROS) and the

activation of a damage-amplifying inflammatory cascade (38,39) (Figure I.4). All these changes result in a microvascular and epithelial dysfunction of the transplanted lung (40,41) with increased permeability of the alveolar membrane and development of lung edema. This is physiologically translated in an increased pulmonary vascular resistance (PVR), ventilation-perfusion mismatch, decreased lung compliance (Compl), intrapulmonary shunting and impaired gas exchange (42).

INFLAMMATION – The inflammatory cascade during IRI is organized in a biphasic response where resident macrophages are the motor of the pulmonary injury. In response to ischemia and/or hypoxia with subsequent upregulation of NADPH oxidase and ROS production, alveolar macrophages can produce a large amount of cytokines and procoagulant factors through NF-KB signaling. NF-KB is the main transcription factor and an important regulator of inflammatory cytokines, chemokines, cell adhesion molecules and apoptosis signals involved in the process (42).

Donor macrophages modulate the early phase of reperfusion injury and form the main initial source of TNF-α, IFN-γ, MCP-1 and IL-8 (43). This is followed by a delayed phase, with massive infiltration of recipient neutrophils upon reperfusion of the donor lung in the recipient's chest. These are able to deliver elastases, proteases and toxic radicals which will further promote oxidative stress signaling and amplification of the pro-inflammatory cascade. Release of pro-inflammatory mediators and upregulation of adhesion molecules will attract more neutrophils and activate T-cells that will further nourish the pro-inflammatory environment of the lung (44–46). Significantly increased levels of pro- and anti-inflammatory cytokines such as TNF-α, IFN-γ, IL-8, IL-2, IL-6, IL-10, IL-12 and IL-18 are measured during both ischemia and reperfusion in the lung (bronchoalveolar lavage fluid (BAL), tissue) and serum of donor/recipient. The origin of these cytokines and chemokines are the inflammatory cells, but also structural cells (epithelial cells and endothelial cells) contribute to this damage-amplifying

pro-inflammatory cascade. IL-8 levels in BAL even correlate with graft function after lung transplantation and are correlated with an increased risk of death from PGD (36). Macrophages also release procoagulant factors such as PAF. Platelet activation leads to formation of micro thrombi and activation of leukocytes to further enhance the inflammatory cascade in the late phase after reperfusion. Progressive microvascular obstruction is mediated by this release of procoagulant factors and the vasoactive effect of inflammatory mediators (47).

ROS - During cold preservation, ischemia and/or hypoxia lead to depletion of ATP-stores and accumulation of ATP degradation products such as hypoxanthine and xanthine (41). The absence of flow and shear stress in the lung upregulates NADPH oxidase and xanthine oxidase which can produce massive amounts of superoxide anion, hydrogen peroxide and hydroxyl radicals (47). These upregulated enzymes can be found in high concentration on dysfunctional endothelial cells, but also on vascular smooth muscle cells, macrophages, neutrophils and platelets (48). ROS can cause direct damage to the pulmonary endothelium and epithelium by interacting with the phospholipids of the cell membrane. Oxidative stress stimulates the transcription of cell-surface adhesion molecules and release of cytokines that will attract and activate inflammatory cells after reperfusion (43).

CELL DEATH – There are two forms of cell death: necrosis and apoptosis. Apoptosis is a relatively quiescent kind of cell death since it does not induce inflammation and cytokine release, as is seen in necrosis. Therefore, apoptosis is referred to as controlled cell death, while necrosis is an uncontrolled disintegration of the cell with release of its content (49).

With prolonged periods of ischemia, the mode of cell death will be primarily necrosis. But for shorter periods of ischemia, the mode of cell death will be primarily apoptosis upon reperfusion of the graft. Therefore, apoptosis is thought to be the main mechanism of cell death observed during pulmonary IRI and increases rapidly after reperfusion with a peak that coincides with the highest caspase-8 levels two hours after reperfusion (42). Apoptosis is triggered by the

intrinsic pathway through stressors like oxidative stress that upregulate pro-apoptotic proteins such as BID and BAX. These proteins of the Bcl-2 family, lead to increased mitochondrial permeability in pulmonary epithelial cells with release of cytochrome c and apoptotic protease activating factor (APAF). Together with caspase-9, they form an apoptosome to activate effector caspase-3 and the subsequent caspase cascade towards cell apoptosis (50). The extrinsic pathway, triggered by activation of specific receptor-ligand signaling interactions through Fas/Fas ligand and TNF receptors, will give rise to a cascade of caspase activation such as caspase-8 (initiator caspase) and caspase-3, -6, -7 (executioner caspases), that ultimately results in DNA fragmentation and cell death (51).

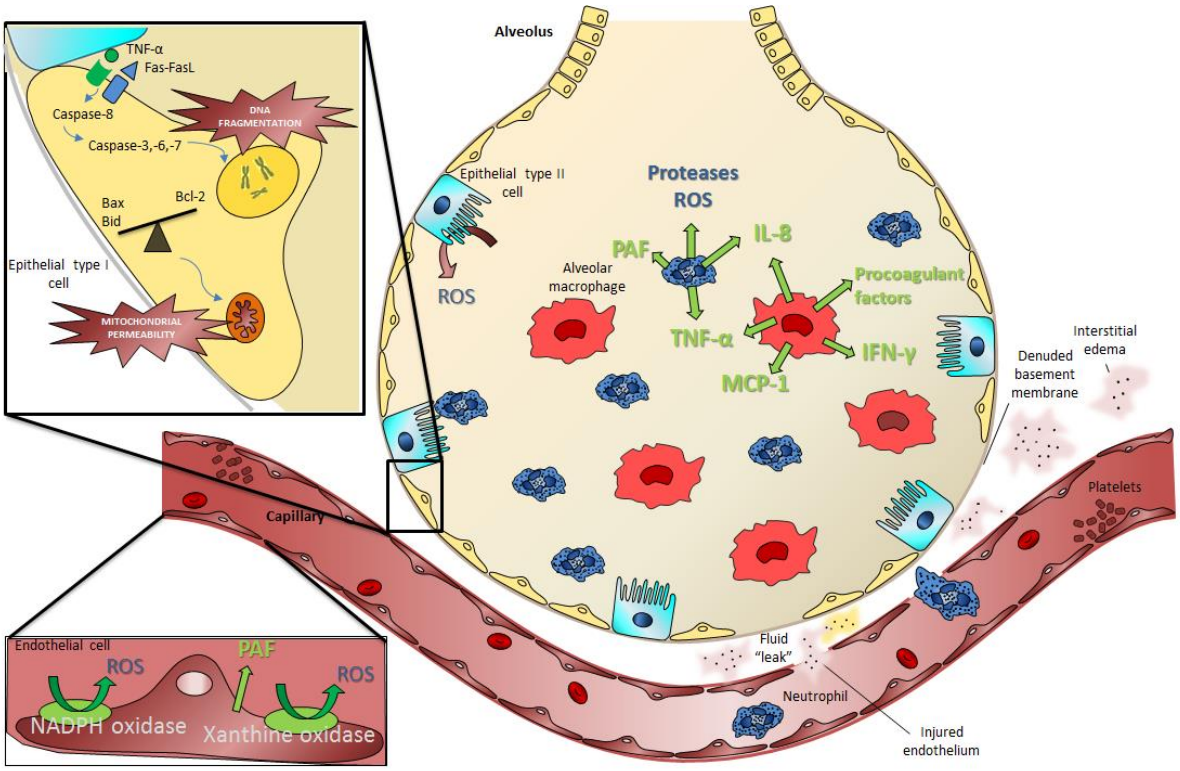


Figure I.4 – Pathophysiology of PGD, dominated by donor macrophages in the early phase and recipient neutrophils in the delayed phase. A complex interplay of inflammation, apoptosis, reactive oxygen species ultimately results in diffuse alveolar damage with capillary leakage, interstitial and ultimately alveolar edema.

Risk factors

There are several known risk factors for the development of PGD. The lung graft can accumulate numerous injuries at various stages during the lung transplant process: during the process of brain death (autonomic-humoral dysregulation) or circulatory death (warm ischemia), procurement and preservation of the donor organ (cold ischemia), reperfusion of the organ in the recipient's chest and during the early postoperative stage after the transplant procedure. Donor smoking (especially > 20 pack years) is associated with the development of severe PGD, but is a controversial topic since the history of donor smoking is often inaccurate (5). Operative-related factors include single-lung transplant, prolonged cold ischemic time, intracellular type preservation solutions, high fractional inspired oxygen upon reperfusion, poly-transfusion and the use of cardiopulmonary bypass. Also recipient-related factors have been linked with an increased risk of developing PGD: overweight and obese body mass index, preoperative sarcoidosis, idiopathic pulmonary fibrosis, primary pulmonary arterial hypertension or increased pulmonary arterial pressures (26,52).

Treatment

Unfortunately, only supportive treatment is available once PGD has been established. This support includes lung-protective ventilation, restrictive fluid balance, inhaled nitric oxide (iNO) and extracorporeal membrane oxygenation (ECMO) as a final salvage strategy (5,53). Also preventive treatment options are not yet definitely proven to be clinically effective. Most clinical trials are based on small sample sizes and there are only a few randomized trials available. Surfactant instillation and iNO seemed promising, but there is a lack of efficiency for their use in clinical practice (54–56). Retransplantation can be considered in highly selective cases, however, predicted survival is poor and therefore retransplantation for severe PGD is not recommended (57). In case gas exchange is the main limiting factor, veno-venous ECMO can be safely chosen over veno-arterial ECMO. However, in case the symptoms progress towards

circulatory failure, veno-arterial ECMO should be considered (58,59). In conclusion, the second major problem identified and addressed in this thesis project will be:

Problem 2: Currently, only supportive treatment options are available for PGD, which still occurs in up to 30% of patients after lung transplantation and limits both short- and long-term outcome.

D) EX-VIVO LUNG PERFUSION

Ex-vivo lung perfusion (EVLP) is a form of isolated lung perfusion in normothermic conditions. It can be achieved with a pump-driven perfusion machine that recirculates a preservation solution through the vasculature of the lung in addition to mechanical ventilation (Figure I.5). It is offered to assess the graft outside of the body, and to serve as an alternative to cold static lung preservation where lungs are stored on ice. Advancements in lung-preservation techniques in the pre-retrieval and post-retrieval periods aim at increasing the pool of available donors, and novel research and discoveries in this area steadily improve post-transplantation adverse events. Ex-vivo lung perfusion (EVLP) is such a novel technique for normothermic lung preservation to improve both number and quality of available donor lungs.

History

EVLP was introduced first by Jirsch in 1970 (60), who used perfused canine lobes in an ex-vivo isolated perfusion model for evaluation of preservation strategies. Initial efforts failed however, due to the inability to maintain the integrity of the alveolar-capillary membrane, resulting in pulmonary edema. In 2001 Steen and colleagues succeeded in performing the first lung transplantation of uncontrolled DCD donor lungs that were evaluated ex-vivo prior to transplantation (61). EVLP was thus initially developed as a platform to carefully evaluate donor lungs to assess their transplantability based on physiological parameters. In 2008, the Toronto group pushed the limits with their technique of prolonged preservation (up to 12 hours) (62). This was a turning point because since then, lungs could be preserved safely for a prolonged period on EVLP. Lately, the interest in EVLP has further developed towards its usage as a platform for active reconditioning of donor lungs (63). Due to technical improvements and efforts of research groups around the world, EVLP could become a validated treatment platform for evaluation, preservation and even reconditioning of donor lungs (63). With EVLP, we aim at maintaining and even improving the quality of the donor organ.

Technique of ex-vivo lung perfusion

During EVLP, lungs are mounted in an isolated system. Therefore, lungs have to be cannulated on ice in preparation for ex-vivo organ perfusion. A perfusion cannula is fixed in the pulmonary trunk. The left atrium can be cannulated with a funnel-shaped cannula or can be left open for drainage (64). Finally, an endotracheal tube is fixed in the trachea. All EVLP-circuits consist of a pump, gas exchanger with heater/cooler, a hard-shell reservoir and most often a leukocyte filter (Figure I.5). The system is primed with an extracellular dextran 40-based solution with optimal colloid pressure (with are without addition of albumin or red blood cells). The solution is further completed with additives (methylprednisolone, antibiotics, and heparin) and corrected for bicarbonate and glucose after biochemical analysis. The target flow during EVLP ranges from 40% to 100% of the estimated cardiac output depending on the protocol that is used. Lungs are ventilated with protective ventilator settings during EVLP, starting when a target outflow temperature is reached (32-34 °C). An overview of all settings in the 3 most used clinical protocols (Lund, Toronto, OCS^{TM}) is provided in Table I.4 (adapted from Van Raemdonck et al. Transpl Int 2014).

Table I.4 – Different EVLP protocols

	Toronto	Lund	OCS^{TM}
Target Flow	40% CO	100% CO	2.0-2.5l/min
Left atrium	Closed	Open	Open
Perfusate	Steen Solution	Steen Solution + RBCs	OCS Solution + RBCs
Ventilation started	32 °C	32 °C	34 °C
Tidal Volume	7 ml/kg	5-7 ml/kg	6 ml/kg
RR (bpm)	7	20	10
FiO_2 (%)	21	50	12
Portable	No	No	Yes

OCS = Organ Care System (Transmedics, Andover, MA, USA); CO = cardiac output; RBC's = red blood cells; RR = respiratory rate; FiO_2 = fraction of inspired oxygen. Adapted from (65)

EVLP allows for careful physiological evaluation of the donor lung. During EVLP, we can measure oxygenation capacity (PaO_2/FiO_2) by sampling the perfusate at the outflow, evaluate lung compliance based on ventilatory parameters (Ppeak, Pplateau, compliance), and calculate pulmonary vascular resistance (PVR) (66) based on pulmonary artery pressure (PAP), left atrial pressure (LAP) and the flow (l/min). But also perfusate samples and bronchoalveolar lavage (BAL) fluid can be analyzed for evaluation of the metabolic status and inflammatory profile of the perfused lung graft (67,68). At 4 hours of EVLP, IL-8 is a good parameter to predict PGD3 development for example (69). However, these analyses are not immediately available so we mainly rely on physiological parameters only.

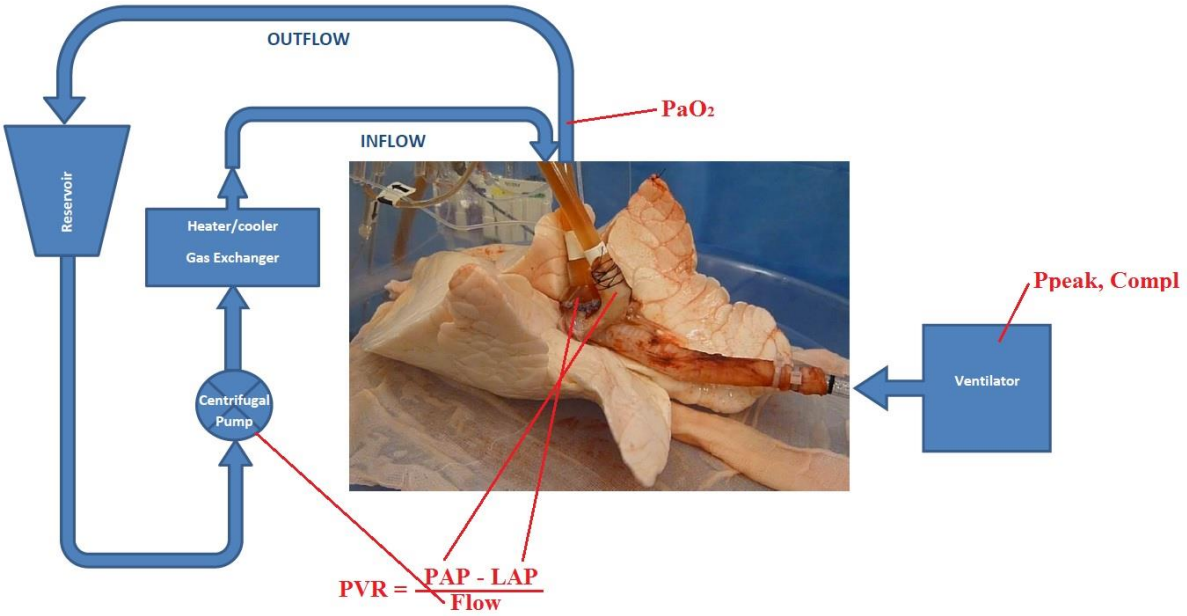


Figure I.5 – A classic EVLP set-up (here depicted with an acellular perfused porcine lung) consists of an inflow cannula inserted on the pulmonary artery, an outflow cannula (or open atrium), a reservoir that collects the perfusate draining out of the lung, a centrifugal (or pulsatile) pump, a heater-cooler system, gas exchanger, and a ventilator.

Clinical experience and early outcome after EVLP lung transplantation

Since the first clinical EVLP case was reported in 2001, many research groups have engaged themselves to use this new technology to increase the donor pool. Pre-clinical studies already showed that normothermic machine preservation could be superior to cold storage preservation

(70). And lately, early clinical experience showed comparable early outcome and incidence of primary graft dysfunction after EVLP (71,72), even when high-risk donors were used (73–75). Several groups reported their experience on transplanted lungs of initially rejected donor lungs after reconditioning ex-vivo (76–79). However, many of these lungs that are considered marginal or initially not transplantable are already transplanted as an extended-criteria donor without the use of EVLP in other centers (20,80). These centers also reported excellent outcome, which could indicate that EVLP is not always necessary to safely transplant marginal donor lungs (23).

It is still not clear what the optimal time interval is to perform EVLP. This can range from a short assessment on EVLP, to a full replacement of the cold preservation time by normothermic preservation. Also, the potential combination with cold storage before and after EVLP is still not clarified. There might be an advantage of limiting the cold storage period to reduce IRI by a portable EVLP device. Early results after portable EVLP show excellent results (72). However, some groups showed better outcome when EVLP was applied after a longer initial cold storage period (81). This might allow safe transportation to an experienced EVLP center that could then evaluate and optimize the donor lung prior to a second cold preservation period during transport to the recipient center (82). From pre-clinical investigations, we know that cold storage after normothermic machine perfusion can safely be applied up to 10 hours (83) which further facilitates logistics for lung transplantation. Some groups even applied an initial cold preservation period prior to normothermic machine perfusion as an ischemic preconditioning strategy, and reported superior graft function (84,85). Vice versa, the portable lung perfusion device can also be used after a longer cold storage period when necessary (86).

EVLP results in an increased use of offered donor lungs with comparable early outcome after lung transplantation. Moreover, not many groups reported the long-term effects of EVLP yet. Tikkanen and colleagues reported acceptable long-term survival, graft function and

improvement of quality of life after ex-vivo lung perfused lung transplantation that is comparable with the conventionally selected and cold-preserved lungs in a case series of 60 EVLP cases (87). In their retrospective analysis, they even found that freedom from CLAD in DBD donors (and not DCD donors) was superior when EVLP was used prior to transplantation. Other investigators also observed this higher freedom from CLAD with EVLP (88).

Reconditioning, a term for quality improvement

Although EVLP was introduced as an evaluation tool to gain more information on questionable donor organs prior to transplantation, it has recently been put forward as an ideal treatment platform for active improvement of lung quality of initially rejected donor lungs. The term "reconditioning" of donor organs is introduced here, referring to any intervention that will restore the organ to become transplantable by improving its quality which was initially unsatisfactory. To improve the number of transplantable donor organs, we define 3 intervals for this potential quality improvement (or reconditioning): preconditioning, perconditioning and postconditioning (Figure I.6).

First, "***pre***conditioning" is defined as any intervention to optimize organ quality while the organ is still inside the donor (before onset of ischemia). This can be considered as classical donor management.

Second, we consider "***per***conditioning" as any strategy applied during the ischemic interval. In practice, this means interventions during cold static preservation on ice.

Finally, treatment interventions can also be applied after reinstallation of organ perfusion ("***post***conditioning"). This could be done in the recipient after transplantation. However, since the lung is normothermically perfused during EVLP, we also consider this technique as a platform for postconditioning without harming the recipient.

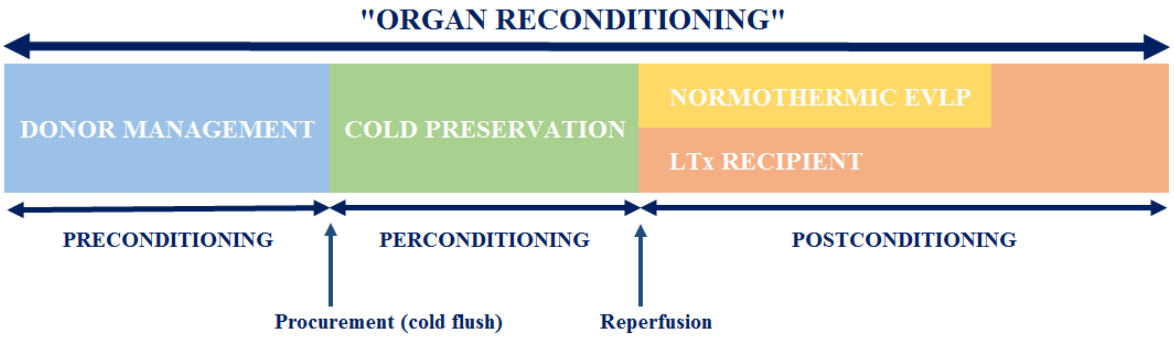


Figure I.6 – Organ reconditioning (improving lung quality) can be done at three different stages: while the organs are still in the donor (preconditioning), during cold ischemic preservation (perconditioning) and while they are reperfused inside the recipient after transplantation (LTx) or on EVLP (postconditioning)

Several clinical case reports of organ reconditioning have been reported. For example, neurogenic lung edema (89) or pulmonary congestion after contusion (90) can be salvaged by perfusing the graft with a high-oncotic perfusate. Fibrinolytics can be used to improve lung physiology and to reduce inflammation (91), and can result in resuscitation of lungs initially rejected due to large pulmonary emboli (92,93). Many anti-inflammatory therapies have been proposed and investigated in experimental settings: surfactant (94,95), ascorbic acid (96), adenosine receptor agonist (97,98), IL-10 vector (99), steroids (100). Also inhaled anti-inflammatory agents have been investigated with promising results in pre-clinical models: hydrogen (101,102), beta2-agonist (103,104). Even microbial load could be reduced in an infected lung during EVLP (105,106). To solve the organ shortage issue for smaller recipients, EVLP could be used for graft downsizing while normothermically perfusing the lung (107). In this way, we could include marginal lungs and make them suitable for transplantation in smaller recipients. However, apart from isolated cases of active lung reconditioning, there are currently no validated treatment options we can use to recondition rejected donor lungs.

In summary, reconditioning is seen as an optimization of donor lung quality which can be achieved inside the donor (preconditioning), during cold preservation (perconditioning) or after reinstallation of perfusion on EVLP or inside the recipient (postconditioning). Experimental reconditioning strategies are needed for clinical translation and should be based on clear mechanistic hypotheses.

Three reconditioning strategies will be investigated in this PhD project, which are selected based on their anti-inflammatory, pro-survival or immunoregulatory characteristics:

1) glucocorticoids, 2) noble gases, and 3) mesenchymal cells.

E) GLUCOCORTICOIDS

Used clinically since the 1920s, corticosteroids interrupt multiple steps in immune activation. They are among the most potent anti-inflammatory and immunosuppressive agents due to their ability to inhibit antigen presentation, cytokine production, and proliferation of immune cells.

There is a vast experience with corticosteroids in the field of transplantation, used as an immunosuppressive drug to prevent rejection and prolong graft survival (108). And also for the management of a brain-dead donor, steroids are often included as an organoprotective treatment strategy. Brain death induces a systemic inflammatory response with release of many pro-inflammatory cytokines such as TNF-α, IL-1, IL-6 and IL-8 that can damage the donor organs. Therefore, blunting this inflammation with early glucocorticoid administration, could decrease cytokine production and may prevent alterations induced by pro-inflammatory mediators, stabilize cell membranes, reduce expression of cell surface adhesion molecules and avoid lipid peroxidation after the ischemic period (109). These actions will theoretically improve donor organ function. In the respiratory system, steroids can bind to the glucocorticosteroid receptors which are ubiquitously expressed in all cells throughout the airways. After translocation to the cell nucleus, they inhibit NFκ-B activation followed by blockage of the pro-inflammatory genes involved in IRI (21–23). It is therefore an ideal drug to improve donor lung quality by reducing IRI inherent to the transplant process.

The CORTICOME study, investigating the effect of low-dose hydrocortisone administration in brain-dead donors, failed to increase organ recovery or early graft function however, early administration of low-dose hydrocortisone did result in hemodynamic stabilization of the donor (110). Therefore, routine steroid administration should be embedded in a multimodal approach of the hemodynamic unstable brain-dead donor. Dhar et al showed that a lower-dose corticosteroid protocol had similar results as high-dose regimens, and donors had an improved glycemic control (111). The beneficial effect of corticosteroids on improved oxygenation,

increased organ recovery and organ function is based on observational studies only (112,113), and large randomized controlled trials are lacking. Current recommendations to use steroids for donor management of brain-dead donors, is therefore based on low-grade evidence (114,115). Besides their potential beneficial role in DBD donation, their role in DCD donation to protect against warm-ischemic injury has never been investigated since the dead-donor rule limits interventional studies in DCDs. Nevertheless, over 90% of centers using DCD organs report that steroids are applied prior to circulatory arrest (8). However, this practice is based on limited data from DCD observational studies only and pre-clinical research should be conducted to support this practice.

We hypothesize that administration of steroids prior to the onset of warm-ischemic injury and during EVLP has a beneficial impact on pulmonary graft function.

F) NOBLE GASES ARGON AND XENON

Argon (Ar) and xenon (Xe), both noble gases, have gathered increased interest as an innovative reconditioning strategy. They have been shown to protect against ischemia-reperfusion injury and hypoxia in various organs including the brain (116–120), myocardium (121,122), vascular endothelium and kidneys (123,124). Despite being described as chemically inert, these noble gases have been repeatedly demonstrated to exhibit biological effects such as anesthesia (125) and inducing improved survival in hypoxic injury models (126). They have unique physico-chemical properties that are summarized in Table I.5. They have the potential to interact with cell survival pathways and are therefore referred to as anti-apoptotic (127,128). Also, the gaseous nature of these components allows unique delivery through the airways in the alveolar spaces. This characteristic, together with its anti-apoptotic properties, makes them excellent candidates to be investigated in lung transplantation to tackle IRI and its clinical end-result, primary graft dysfunction. The main advantage in the search of a therapeutic intervention to attack pulmonary IRI, is that lungs can be approached at the epithelial side since the airways are continuously exposed to the environment. Therapeutics can therefore be delivered through distribution via bronchoscopy (instillation of therapeutics) or through ventilation (inhaled therapeutics). But also the conventional distribution via the vasculature of the lung can be chosen (saturation of perfusion solution).

Table I.5 Physico-chemical properties of noble gases Ar and Xe

	Argon	Xenon
Atomic number	18	54
Atomic mass	39.948	131.30
Melting point (K)	83.78	161.3
Boiling point (K)	87.29	166.1
Ionization potential (kJ mol^{-1})	1520	1170
Density (g cm^{-3})	1.784	5.88
Color of spectrum	Violet	Blue Green
Blood/gas partition coefficient	0.037	0.140

We know from electrophysiological studies that the neuroprotective effect of Xe arises from an inhibition of the N-methyl-D-aspartate receptor and activation of the TREK-1 channels (129). NMDA-receptor inhibition also results in its anesthetic effect (130), together with enhancing signal conduction of the GABA receptors (131,132). The organoprotective effects of Xe found in myocardial and renal ischemia models, are mediated by pro-survival signals that target MAPK and ERK-1/2 pathways (133,134), HIF-1α activation (135) and mitochondrial permeability (136). However, the mechanism of action for Ar is not yet unraveled (137). There is growing evidence that the ERK-1/2 signaling cascade is involved (138) but how the modulation of this signaling pathway might lead to organ protection, is not yet unraveled (139).

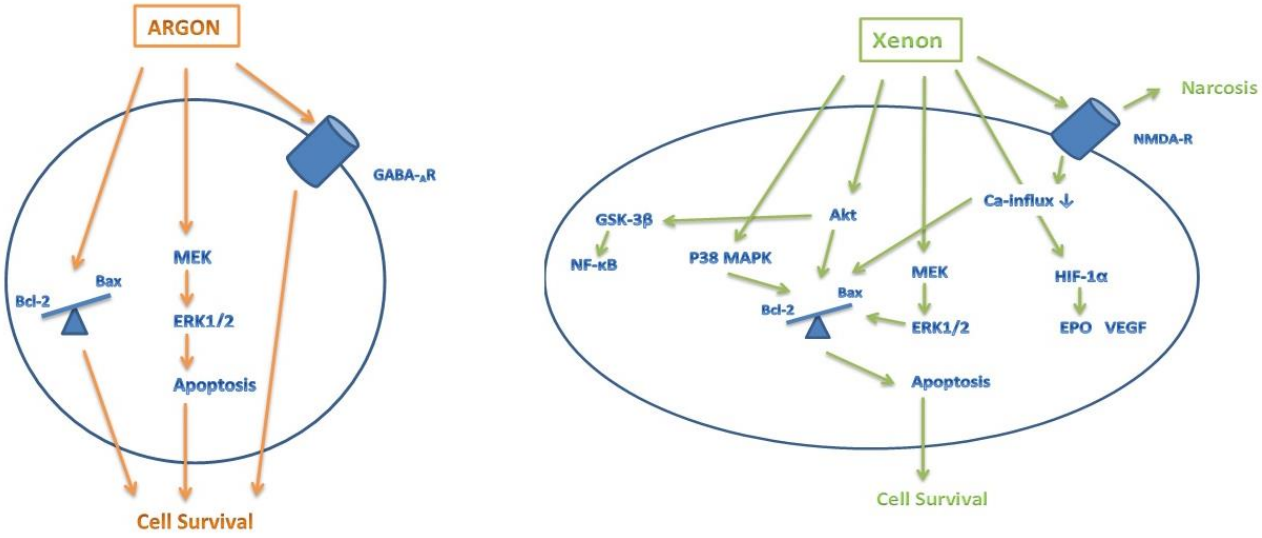


Figure I.7 Summary of the possible pro-survival working mechanisms of Ar and Xe to attenuate IRI.

We hypothesized that modulation of survival pathways and ischemic injury by noble gases might improve pulmonary graft quality prior to lung transplantation.

G) BONE-MARROW DERIVED MESENCHYMAL CELLS

There is a growing interest in bone-marrow derived cell products as an innovative reconditioning strategy for acute organ injury for different reasons (140). First of all, they can differentiate into cells of different germ layers (141) and can produce trophic factors to stimulate resident progenitor cells for tissue repair and organ function recovery (142–144). Therefore, they are currently being investigated for improving neurological outcome after ischemic stroke (145) and improving cardiovascular function after acute myocardial infarction (146). Secondly, mesenchymal cell products have been shown to have immunoregulatory capabilities by mechanisms that have yet to be elucidated. They are known to modulate T-cell alloreactivity (147,148) and are therefore of interest in the treatment of immune and inflammatory disorders such as graft-versus-host disease (149,150), inflammatory bowel disease (151) and organ transplantation (152,153). Whether these immunoregulatory properties might also avoid chronic rejection in solid organ transplantation with low or no immunosuppression remains a topic of future studies (154,155). No large well-designed trials are performed yet, and data of their immunoregulatory capabilities is mainly based on in vitro studies, therefore it is too early to know whether these therapeutic cell interventions can markedly improve outcome in well-selected patients (156). Results of the first Phase I and II trials do indicate that administration of mesenchymal cells is safe. However, efficacy is yet to be proven in Phase III trials (155).

Also in the lung, mesenchymal cell products have been investigated in the setting of acute lung injury (157–159) and acute respiratory distress syndrome (160), which share common pathways with the pathophysiology of PGD. More specifically, pilot studies show that they can reduce IRI inherent to solid organ transplantation in the lung (161–163), but also in other organ systems (164,165). Here, their beneficial effects probably result from paracrine mechanisms and cell-cell interaction rather than engraftment and repair of diseased tissue (166). An altered

inflammatory balance, with a decrease in pro-inflammatory and increase in anti-inflammatory cytokines, has been observed (161). In hypoxic conditions, such as ischemic injury models, secretion of growth factors such as VEGF and ANG-1 can stimulate angiogenesis and tissue repair (163,167).

Mesenchymal stem cells and multipotent adult progenitor cells

The Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy defined human mesenchymal stem cells (MSC) using 3 minimal criteria. They must be adherent to plastic in standard culture conditions, must have a specific phenotypic profile based on positive (CD105, CD73, CD90) and negative (CD45, CD34, CD14 or CD11b, CD79α or CD19, HLA-DR) markers and must be able to differentiate in vitro into osteoblasts, adipocytes and chondroblasts (168). In 2002, a promising new mesenchymal cell type, the multipotent adult progenitor cell (MAPC) has been characterized. The MAPC has an important advantage over the MSC: they have a lower senescence than MSCs and can be replicated >70 times, which allows banking of large amounts of cells and therefore a high potential for clinical applications. MAPC cells are now considered to be a distinct cell population (169) with a specific phenotypic profile (170). Both the MSC and the MAPC are bone-marrow derived cells with many similarities, but due to different culture conditions they adopt different phenotypes (169).

The interest in MAPC cellular therapy in pathologies such as GVHD, chronic rejection in solid organ transplantation, inflammatory bowed disease, etc is based on the immunoregulatory effect of MAPC on the adaptive immunes system. That is, MAPC cells exert a strong immunosuppressive effect on T-cel proliferation, mediated by release of soluble factors such as IDO (147). However, the immunological mechanism of PGD mainly involves the innate immune system since the adaptive immune response is characterized by low kinetics and is only activated after a few days. Only limited in-vitro data of the effect of MAPC on the innate

immune system is available. MAPC cells are known to suppress the cytolytic activity of NK-cells and induce shifting from M1 to M2 macrophage phenotypes by inhibiting release of pro-inflammatory markers such as MMP-9 (171). An effect on neutrophils, the most important effector cell in the pathophysiology of PGD, has not been described so far. In our study, investigating new strategies to tackle PGD, we will focus on the impact of MAPC administration on the innate immune system which is largely unexplored so far.

We hypothesized that the immunoregulatory properties of MAPC might form a mechanistic basis for improving pulmonary graft quality prior to lung transplantation.

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CHAPTER II

RATIONALE AND AIMS

The success of lung transplantation is critically dependent on the number and quality of donor organs. This is currently one of the main challenges to maintain and to improve the outcome for patients on the waiting list and recipients after transplantation. Therefore, the ***overall aim*** of this project is to provide insights in donor organ shortage and to develop strategies to increase the number and quality of available donor lungs to improve early outcome after lung transplantation.

We identify two sequential time intervals prior to the lung transplant procedure that determine the number and quality of the pulmonary graft (Figure II.1). First, during the ***donor phase***, a suitable donor has to be offered and selected for organ transplantation and donor organs have to be carefully evaluated in the donor chest or even be treated to improve their quality. Second, storage conditions during the ***preservation phase*** further define the quality of the lung graft. Organs can undergo an additional evaluation outside the donor's chest during ex-vivo lung perfusion or can even be reconditioned prior to transplantation.

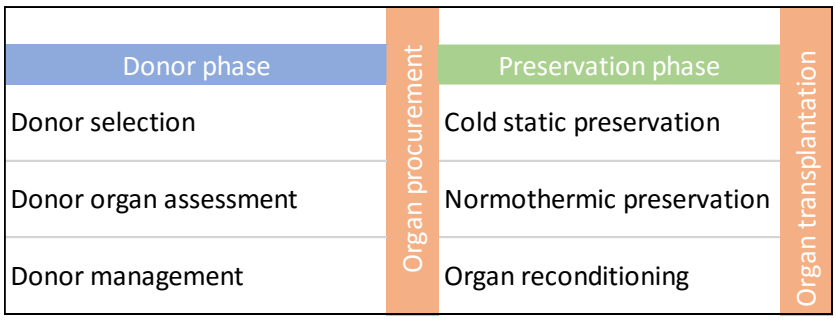

Figure II.1 – Two sequential time intervals where strategies can be implemented to increase both number and quality of available donor organs: the donor phase and preservation phase.

Lung transplantation is a challenging process and its success is already partly determined in the donor phase. We will investigate how advancements during the donor phase can increase both number and quality of transplantable donor lungs (CHAPTER III). First of all, we will discuss a strategy of **evaluating** more **organs** (both in-vivo and ex-vivo) to increase the number of transplantable donor organs (CHAPTER III.A). Secondly, we aim to provide evidence to

support the practice of pre-arrest **DCD donor treatment with steroids** to improve the quality of the donor lung (CHAPTER III.B).

In CHAPTER IV, we will investigate if EVLP is a useful strategy to increase the number of available donor organs during the preservation phase. In CHAPTER IV.A we aim to investigate the **potential of EVLP** to increase the number and quality of donor lungs by a retrospective analysis of our donor database data. By providing more information on donor lungs that are currently not used in our own transplant center, we will identify and calculate which donor organs can be salvaged by EVLP in the future. In CHAPTER IV.B the feasibility of **EVLP implementation** in clinical practice will be demonstrated by a case report of a pediatric combined liver-lung transplantation where the lungs were preserved on EVLP while the liver was implanted first.

Reconditioning or actively improving lung graft quality, can be pursued by implementing strategies prior (***pre***conditioning), during (***per***conditioning) or after (***post***conditioning) injury to the donor organ.

In CHAPTER V, we will investigate reconditioning strategies with the **noble gases** argon (Ar) and xenon (Xe). To protect the alveolar-capillary wall integrity during lung preservation, a postconditioning effect (post-injury) of Ar and Xe on warm-ischemic injury will be investigated ex-vivo (CHAPTER V.A). In addition, also prolonged exposure to Ar to protect against ischemia-reperfusion injury will be investigated (CHAPTER V.B). In this chapter, the effect of exposure to Ar prior (preconditioning), during (perconditioning) and after (postconditioning) a cold-ischemic insult will be investigated.

Finally, in CHAPTER VI, **multipotent adult progenitor cells** (MAPC, bone-marrow derived mesenchymal cells) will be investigated as an innovative reconditioning strategy to improve donor organ quality prior to lung transplantation by immunoregulatory mechanisms. In CHAPTER VI.A, the ideal route of administration (intravenous or intratracheal) will be

investigated in a porcine ex-vivo model of warm ischemia. The most potent administration route will be further investigated with a higher dose of MAPC to reveal a beneficial effect on the inflammatory profile of the donor lung (CHAPTER VI.B).

In summary, we aim:

- To investigate how advancements during the ***donor phase*** can increase the number and quality of transplantable donor lungs (CHAPTER III).
- To investigate the ***potential and feasibility of EVLP*** as a platform to increase the number and quality of transplantable donor lungs during the ***preservation phase*** (CHAPTER IV).
- To investigate the role of ***noble gases as a reconditioning strategy*** to improve donor quality by better preserving the alveolar-capillary wall integrity (CHAPTER V).
- To investigate the role of ***multipotent adult progenitor cells as a reconditioning strategy*** to improve donor organ quality by an immunoregulatory effect (CHAPTER VI).

CHAPTER III
DONOR ASSESSMENT AND TREATMENT

III.A IN-SITU DONOR LUNG EVALUATION TO MAXIMIZE THE LUNG YIELD

Adapted from:
Martens A, Neyrinck A, Van Raemdonck D. Accepting donor lungs for transplant: let Lisa and Bob finish the job! Eur J Cardiothorac Surg. 2016 Nov;50(5):832-833
(DOI: 10.1093/ejcts/ezw261)

A) PREFACE

Until today, assessment of donor lung quality is still based on a limited amount of donor data in combination with in situ inspection of the lungs by the procurement team. Therefore, EVLP was introduced in 2001, as a method for additional evaluation of lung graft quality in a donor of unknown quality (1). Large randomized controlled clinical trials have demonstrated that normothermic EVLP for standard-criteria donor (SCD) lungs is safe, and holds similar short-term outcome for patients receiving a donor lung that was preserved on ice (2). More recently, clinical trials are looking into recruiting more extended-criteria donor (ECD) lungs with EVLP to enlarge the donor pool (3). ECD lungs are donor lungs not matching the strict criteria of the ideal lung donor. Many centers report similar long-term outcome for ECD lung transplantation compared to SCD transplantation. However, early outcome is negatively affected with increased duration of mechanical ventilation, prolonged ICU stay and higher incidence of primary graft dysfunction (PGD) (4). Careful evaluation of these ECD lungs is therefore pivotal. With this reason, retrieval teams are sent out more often to recruit these extended-criteria donor lungs for EVLP. However, only a minority of extended-criteria donor lungs undergo an additional evaluation on EVLP in the end, and still, transplantation of ECD lungs seams safe and has similar long-term outcome compared to SCD lungs (5).

In many transplant centers, donor organ yield has increased due to careful in-situ evaluation of the lungs in the donor chest. And thus, by merely evaluating lung quality in the donor chest more often, instead of relying on registered donor data only, we can recruit and transplant more donor organs.

We have addressed this observation in an editorial comment on an original article of donor lung assessment with pulmonary venous blood gases in the donor chest (6).

B) EDITORIAL COMMENT

LISA and BOB are acronyms for *l*ook *i*nside *a*lways and *b*ottom *o*f the *b*asket. These terms are taught during training of new cashiers working in shopping centers to thoroughly check bags. "*You don't always have to spend thousands of dollars on security measures to prevent shoplifting. Taking just a few minutes a day to train your employees on simple tactics to thwart a theft will ultimately make your store much more profitable"* (7). Similarly, our transplant fellows Lisa and Bob when going out on donor runs, should be taught to always look inside the chest cavity themselves and recruit the basal parts when assessing donor lungs.

The selection of donor lungs for transplantation has always been a very subjective process (8). Beside interpretation of chest X-ray and bronchoscopic findings, oxygenation challenge with lungs ventilated on an inspired oxygen fraction (FiO_2) of 1.0 and 5 cmH_2O positive end-expiratory pressure (PEEP) remains a key parameter to judge transplant suitability prior to organ recovery. In the early days of lung transplantation, a lower limit for the ratio of partial arterial oxygen pressure PaO_2/FiO_2 of 300 mmHg was arbitrarily chosen as the standard value for acceptance. This cut-off point, however, was not based on randomized trials and even not on comparative case series studying outcome between patient groups transplanted with donor lungs that fall above or below this value (9). PaO_2 in the donor is often measured on a sample taken from a radial artery catheter. It is well known that this value may differ from samples taken more centrally and does not always reflect the true oxygenation capacity of the donor lungs. An arterial blood sample also reflects gas exchange in both lungs and does not provide any information on each individual lung. Previous studies have demonstrated that gas analysis on a sample taken from the pulmonary veins may better reflect the true oxygenation capacity of each lung separately (10,11). One study reported that differential pulmonary vein gases better predicted primary graft dysfunction after lung transplantation when compared to donor arterial blood gas measurements (12).

The article by Costa and co-workers from Columbia University Medical Center in New York, NY published in this issue of the European Journal of Cardiothoracic Surgery represents the largest reported single-center series so far on the use of selective pulmonary vein gas analysis in donor lung assessment (6). In a series of 259 brain-dead donors, the last PaO_2 in the intensive care unit (ICU) poorly correlated with the intraoperative central PO_2 taken in the aorta prior to cold flush. The investigators have found that intraoperative aortic PO_2 had a high sensitivity (88%), but a low specificity (24%) with a high false positive rate of 76% when compared to the last gas sample taken in the ICU. The balanced accuracy of this test was only 56% indicating that many suitable lungs may not be offered with the donor still being in the ICU if low PaO_2 on its own is considered as an absolute contraindication for transplantation. In addition, blood samples were taken from left and right pulmonary veins and correlated with intra-operative central aortic PO_2, bronchoscopic findings and visual assessment of lung collapse upon temporary disconnection from the ventilator during organ retrieval. Similar findings of high sensitivity, low specificity, high false positive rate, and limited accuracy for central aortic PO_2 were seen when this was correlated with right and left pulmonary vein PO_2. PO_2 in both right and left pulmonary vein was significantly associated ($p<0.001$) with findings at bronchoscopy, palpation, and visual assessment of lung collapse. The authors concluded that pulmonary vein gases will provide a more objective measure for the retrieving surgeon, allowing for more accurate assessment of individual lungs for transplant suitability.

The level of arterial oxygen tension largely depends on the degree of shunt flow through atelectatic or consolidated lung areas. The central arterial PO_2, therefore, reflects a value for both lungs together and may be falsely low or high depending on shunt flow in individual lungs or lobes. Appropriate ventilatory management of a brain-dead donor in the ICU with a small tidal volume (6-8 ml/kg predicted body weight), high PEEP (8 to 10 cmH_2O), and continuous positive airway pressure during the apnea test and recruitment maneuvers is of paramount

importance to improve and maintain oxygenation capacity. In a study by Miñambres and colleagues, aggressive donor management resulted in an increase in lung yield with similar outcome (13).

Sticking to ideal criteria will result in approximately 15-20% of donors having lungs suitable for transplant (8). Current evaluation of donor lung quality at the time of offer is often challenging and quite subjective as gas exchange, chest radiograph, and bronchoscopy may be assessed by personnel not familiar with lung transplantation practice and not being aware of results when using lung allografts from non-ideal, so called extended-criteria donors. Therefore, taking into account transportation costs for a retrieval team to arrive at the donor hospital, lungs should be inspected and evaluated inside the donor whenever possible prior to declining the offer. Gas exchange should be re-evaluated with the chest open and lungs fully ventilated after recruitment of atelectatic zones. It is interesting to see that with increasing practice of ex-vivo lung perfusion (EVLP), many more questionable donor lungs are now being accepted for immediate transplantation after arrival of the retrieval team in the donor hospital with no further need for assessment with EVLP when back in the recipient hospital. The lesson we should remember from this paper by the Columbia University transplant group is the following: “in order to maximize the yield of donor lungs for transplantation, a retrieval team should be sent to the donor hospital for in-situ evaluation whenever possible; let Lisa and Bob finish the job”.

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CHAPTER III
DONOR ASSESSMENT AND TREATMENT

III.B PRE-ARREST DONOR TREATMENT WITH STEROIDS IMPROVES LUNG GRAFT FUNCTION

Adapted from:
Martens A, Boada M, Vanaudenaerde BM, Verleden SE, Vos R, Verleden GM, et al. Steroids can reduce warm ischemic reperfusion injury in a porcine DCD model with EVLP evaluation. Transpl Int. 2016 Nov; 29(11):1237-46
(DOI: 10.1111/tri.12823)

A) PREFACE

In the previous chapter, we have shown that the number of transplantable donor lungs can be increased by careful in situ inspection of the lung graft. In this chapter, we will provide experimental data on a strategy to improve not only the number, but also the quality of the lung graft for transplantation. Donor management is an intensive task at the intensive care unit, aiming at stabilizing the brain-dead donor and attenuating the systemic inflammatory response that coincides with brain death. Forasmuch, current recommendations advice to implement corticosteroids in the management of brain-dead donors. For donors who die after circulatory arrest, such recommendations are non-existent since the dead-donor rule impedes any pre-mortem intervention. Nevertheless, over 90% of centers using DCD organs report that steroids are applied prior to circulatory arrest in their center. However, this practice is based on limited data from DCD observational studies only. Therefore, we aimed at providing pre-clinical evidence to support this practice of pre-arrest DCD donor treatment with steroids to improve organ quality prior to transplantation.

B) ABSTRACT

Donation after circulatory death (DCD) is being used to increase the number of transplantable organs. The role and timing of steroids in DCD donation and ex-vivo lung perfusion (EVLP) has not been thoroughly investigated. In this study, we investigated the effect of steroids on warm-ischemic injury in a porcine model (n=6/group). Following cardiac arrest, grafts were left untouched in the donor (90 min warm ischemia). Graft function was assessed after 6 hours of EVLP. In MP-group, 500 mg methylprednisolone was given prior to cardiac arrest and during EVLP. In CONTR-group no steroids were added. Median lung compliance (13 ml/cmH_20) was significantly better preserved in MP-group than in CONTR-group (30.5 ml/cmH_20). Also, median wet-to-dry-weight (6.11 vs 6.94) and CT-density (182.5 vs 352.9 g/L) were significantly better in MP-group than in CONTR-group, respectively. There was no difference in oxygenation and pulmonary vascular resistance. Perfusate cytokine analysis showed a significant reduction in IL-1β, IL-8, IFN-α, IL-10, TNF-α and IFN-γ in MP-group. Cytokines in bronchoalveolar lavage were not decreased except for IFN-γ. We demonstrated that warm-ischemic injury in DCD donation can be attenuated by steroids when given prior to warm ischemia and during EVLP. Ethical context of donor preconditioning should be discussed further.

C) INTRODUCTION

Lung transplantation remains the only life-saving treatment option for patients suffering from end-stage pulmonary disease. Due to its success, lung transplant programs worldwide are increasing with over 4000 lung transplant procedures performed annually (1). However, there is an on-going disparity between the number of patients on the waiting list and the number of good quality donor organs for transplantation. This leads to increased waiting times and a persistent mortality on the waiting list as high as 15% (2).

Because of this organ shortage, other sources of organ recovery besides the classical brain-dead donor (DBD) are being addressed nowadays. Over the years, organ donation after circulatory death (DCD) has been re-introduced in transplant programs with a marked increase since 2001 (3–5). On average only 7% of all lung grafts are derived from DCD donors according to the latest registry analysis; however in some programs this reaches up to 32% (3). Generally, DCD donation is classified into two main categories and further subdivided following the Maastricht classification (6). In uncontrolled DCD donation the patient is found with circulatory arrest, is dead upon arrival or dies after unsuccessful resuscitation. Controlled DCD donors represent patients who die after a switch-off of mechanical, ventilated or organ-perfusion supported therapies or when circulatory arrest occurs prematurely in a DBD donor. To optimize lung preservation and limit the impact of warm ischemic injury, several strategies have been explored. Topical cooling by insertion of chest tubes can be applied in the controlled setting, but is mostly used in uncontrolled DCD donation (7,8). In controlled donation the current clinical practice includes rapid flush perfusion (antegrade in the donor and retrograde at the back-table after organ recovery) (9). Pre-arrest interventions are mainly limited to heparinisation if legally authorized. This can theoretically be beneficial as confirmed by experimental data (10). However, clinical studies comparing strategies with and without heparin are lacking. In fact, several centers report good outcome without pre-arrest

heparinisation (11). These centers do use a retrograde flush in its heparin-free scenario which also seems protective (12).

In all scenarios, DCD organs suffer from a variable period of warm ischemia which could lead to increased ischemia-reperfusion injury and a reduction in organ quality. The tolerable length of this warm-ischemic interval for lungs can be extended up to 60-90 minutes (13,14). To assess organ quality of lungs donated by a DCD donor prior to transplantation, ex-vivo lung perfusion (EVLP) has been developed (15). With this technique of machine perfusion, lungs are perfused by a pump and ventilated under normothermic conditions. During EVLP, lungs can be physiologically evaluated and nowadays new imaging techniques can even be applied to fully assess the organ of previously unknown quality (16). Since the introduction of EVLP in 2001 by Steen et al (17) there is the actual potential to evaluate the donor organ prior to transplantation. This is especially recommended in uncontrolled DCD donation programs where outcomes are better when EVLP is applied (8). Currently, in only 12% of controlled DCD, EVLP is clinically applied (3). Some groups do report better outcome in controlled DCD donation (18) when lungs have been perfused and evaluated on EVLP. Therefore, it may be advisable to consider this technique as a platform to assess the risk for severe ischemia-reperfusion injury (IRI) to decide on the optimal preservation strategy based on physiological evaluation. This opens up the ability to even recondition donor lungs of unknown or inferior quality prior to transplantation. Hereby, an increase in available donor organs with optimal quality is expected (19,20).

Steroids are among the most potent anti-inflammatory and immunosuppressive agents. In the airways, they bind to the glucocorticosteroid receptors which are ubiquitously expressed in all cells throughout the airways. After translocation to the cell nucleus, they inhibit nuclear factor kappa B (NFκ-B) activation followed by blockage of pro-inflammatory genes (21–23). Therefore, steroids are of particular interest in ischemia-reperfusion injury remodeling. They

are already a component of the perfusate used in the majority of EVLP protocols (15). However, the exact role of steroids during EVLP has never been elucidated and comparative data of EVLP with- and without methylprednisolone is lacking. Besides ex-vivo administration of steroids, these immunomodulatory drugs can also be administered to the donor. Most brain-dead patients are now treated with steroids before procurement of the organs. The rationale to add steroids in the donor is to block the upregulation of several pro-inflammatory cytokines during the onset of brain death and improvement of hemodynamic instability following adrenal insufficiency (24). The evidence, however, is not robust based on a recent meta-analysis (25). In addition, besides these potential benefits in DBD donors, their role in warm-ischemic injury and DCD donation has never been investigated. Nevertheless, over 90% of centers using DCD organs report that steroids are applied prior to circulatory arrest (3).

We aimed to investigate the role of steroids in DCD lung donation to protect against warm ischemia-reperfusion injury. Therefore, in this study, we hypothesized that administration of steroids prior to onset of warm ischemia and during EVLP has a beneficial impact on pulmonary graft function.

D) METHODS

This experimental study was performed in compliance with the Principles of Laboratory animal care published by the National Institute of Health Volume 25, No. 28 (revised 1996). Local ethics approval was obtained at the research institute (NTS P043/2014).

Donor procedure

Domestic pigs Topig 20 (mean 40.75 kg) were divided into 2 groups (n=6/group). Animals were anesthetized with an intramuscular injection of 5 mg/kg Zoletil 100® (Virbac, Carros, France) and 3 mg/kg Xyl-M® 2% (VMD, Arendonk, Belgium). Anaesthesia was maintained using 10 mg/kg/h propofol, 20 µg/kg/h fentanyl and intermittent boli of pancuronium 2 mg for muscle relaxation. Animals were intubated with a 7.0 mm endotracheal tube and ventilated (Aestiva 3000; GE Healthcare Europe GmbH, Little Chalfont, UK) with a tidal volume (TV) of 8 ml/kg, positive end-expiratory pressure (PEEP) of 5 cmH_2O and FiO_2 of 30%. Respiratory rate (RR) was adjusted to the end-tidal carbon dioxide ($ETCO_2$) (45-55 mmHg). Blood pressure was monitored invasively in the right carotid artery. All animals died of cardiac arrest which was induced by direct electrical stimulation of the myocardium with an electrical pulse generator that led to ventricular fibrillation. Animals were disconnected from the ventilator when cardiac arrest was induced. Prior to cardiac arrest, all animals were heparinized with 300 IU/kg. In group 1, 500 mg Solu-Medrol (Pfizer, Brussels, Belgium) was given prior to induction of ventricular fibrillation (MP-group). In group 2, no steroids were administered to the donor animal (CONTR-group).

Following cardiac arrest in the donor, grafts were left untouched in the deceased donor for 90 minutes after which they were flushed antegradely with 50 ml/kg cold thromethamol-buffered OCS Solution (Transmedics, Andover, USA). The heart-lung block was excised and a retrograde flush (1 L thromethamol-buffered OCS solution) was performed at the back table. Lungs were instrumented on ice for a short period of time (73.2 ± 7.5 min) while the XVIVO

(Göteborg, Sweden) cannulas were secured in the pulmonary artery and atrial cuff. An 8.0 mm ET tube was secured in the trachea. The donor procedure was performed as previously described (26).

Ex-vivo lung perfusion

After a 1-hour rewarming period and slow increase of the flow to 40% of the estimated cardiac output (calculated as 100 ml/kg), lungs were further perfused and evaluated for 6 hours in total. Lungs are perfused with an acellular albumin containing dextran solution. The production of the perfusate and technique of EVLP are performed as described previously (26). In the CONTR-group, no steroids were added to the perfusate. In the MP-group, 500 mg Solu-Medrol® (Pfizer, Brussels, Belgium) was added to the perfusate to continue the steroid-exposure to the preconditioned grafts in the MP-group in order to investigate the maximal effect of steroids to DCD-grafts.

During 6 hours of EVLP we monitored dynamic airway compliance (Compl), oxygenation (PaO_2/FiO_2) and pulmonary vascular resistance (PVR) hourly. We analyzed end-experimental parameters only to dichotomize between acceptable and non-acceptable lungs.

Tissue sampling

At the end of the experiment, tissue samples were taken for histological evaluation and wet-to-dry-weight (W/D) ratio calculation (after 48 hrs in the oven at 80°C). Pathology samples are scored by a blinded pathologist for neutrophilia, congestion and presence of eosinophils. Bronchoalveolar lavage with 2 times 30 cc saline 0.9% was performed in the right middle lobe. Pooled fractions were returned and the supernatant was analyzed with a porcine multiplex ELISA kit for IL-1β, IL-4, IL-8, IL-10, IFN-γ, IFN-α and TNF-α according to the manufacturer's protocol (Thermo Fisher Scientific Inc, Massachusetts, USA). Also perfusate samples from the end of the experiment were analyzed with the same ELISA analysis. The left lung was inflated at 25 cmH_2O, frozen solid in the fumes of liquid nitrogen and scanned with

Siemens Somaton CT scanner (Siemens Healthcare, Erlangen, Germany). Lung mass, volume, and density were measured on the basis of the CT-scan, using imaging software (Horos^{TM}) in which the lung is manually delineated and the number of voxels and mean density of the voxels within the volume is determined (27).

Statistical analysis

All data are expressed as median with IQ range when depicting physiological variables in time or as a scatter plot with median and IQ range when comparing variables at the end of the experiment (GraphPad Prism 4, GraphPad Software Inc, La Jolla, USA). Permutation tests were conducted in R (R Foundation, Vienna, Austria) using the "coin" package to compare data at the end of EVLP. Baseline parameters of the donor animals are described as median (25% QI – 75% QI) and are analyzed with the same permutation test.

In cases where lungs could not sustain the full 6 h of EVLP, data points recorded in the next hours after the premature end of EVLP were considered the same as the last data point available to allow comparison at all evaluation points. Therefore, at the end of EVLP the last available data point is included for the statistical analysis. Graft survival on EVLP is analyzed with a log rank test in GraphPad Prism 4 (GraphPad Software Inc, La Jolla, USA).

E) RESULTS

Groups

Baseline parameters are illustrated in Table III.1.

Table III.1 – Baseline parameters of the donor animals.

	CONTR	MP	p-value
Donor			
Weight (kg)	43 (39 - 46)	40 (39 - 46)	0.05
TV (mL/kg)	7.9 (7.7 - 8.0)	7.9 (7.8 - 8.0)	0.79
HR (bpm)	102 (88 - 119)	100 (72 - 114)	0.46
MAP (mmHg)	92 (81 - 103)	86 (72 - 101)	0.43
Compl (ml/cmH_2O)	28.5 (27.0 - 31.0)	30 (27 - 33)	0.45
PaO_2/FiO_2 (mmHg)	427 (410 - 452)	453 (413 - 495)	0.22
Hct (%)	33.7 (31.3 - 35.9)	35.3 (33.3 - 39.9)	0.14
WBC (10^9/L)	14.5 (12.4 - 17.3)	18.2 (11.8 - 22.4)	0.26
Neutrophils (%)	37 (24 - 43)	41 (32 - 59)	0.23
Neutrophils (10^9/L)	5.9 (3.0 - 6.6)	6.5 (5.3 - 10.4)	0.12
CIT (min)	77 (70 - 85)	70 (62.5 - 74.5)	0.06

Data are presented as median (25% - 75% IQR); p-value permutation test.
TV = tidal volume; HR = heart rate; MAP = mean arterial pressure; Compl = Dynamic airway compliance; PaO2/FiO2 = partial oxygen pressure over fractional inspired oxygen concentration; Hct = hematocrit; WBC = white blood cell count; CIT = cold-ischemic time

Functional assessment of pulmonary grafts during EVLP

Figure III.1A depicts the change of dynamic airway compliance over time (median ± IQR). It is similar at the onset of evaluation (starting after 1 hour of EVLP). After the first recruitment maneuver at 1.5 hrs perfusion, compliance increased in both groups followed by a gradual decrease. When comparing the dynamic airway compliance at the end of EVLP (Figure III.1B), we noted that it was significantly better preserved in the MP-group (median Compl 13.0 ml/cmH_20 in CONTR vs 30.5 ml/cmH_20 in MP-group; p=0.03).

Figure III.1C depicts the change in oxygenation (evaluated by PaO_2/FiO_2) over time (median ± IQR). PaO_2/FiO_2 decreased in both groups and was also not significantly different when

comparing it at the end of the experiment (median PaO_2/FiO_2 487 in CONTR vs 430 in MP-group; p=0.59) (Figure III.1D).

Figure III.1E depicts the change in pulmonary vascular resistance (PVR) over time (median ± IQR). PVR is low in both groups at the first evaluation moment (1 hr perfusion). During the experiment, PVR slowly increases in both groups. When comparing PVR at the end of EVLP (Figure III.1F), we observed a similar PVR in both groups (median PVR 474 dynes*s*cm^{-5} in CONTR vs 430 dynes*s*cm^{-5} in MP group; p=0.82).

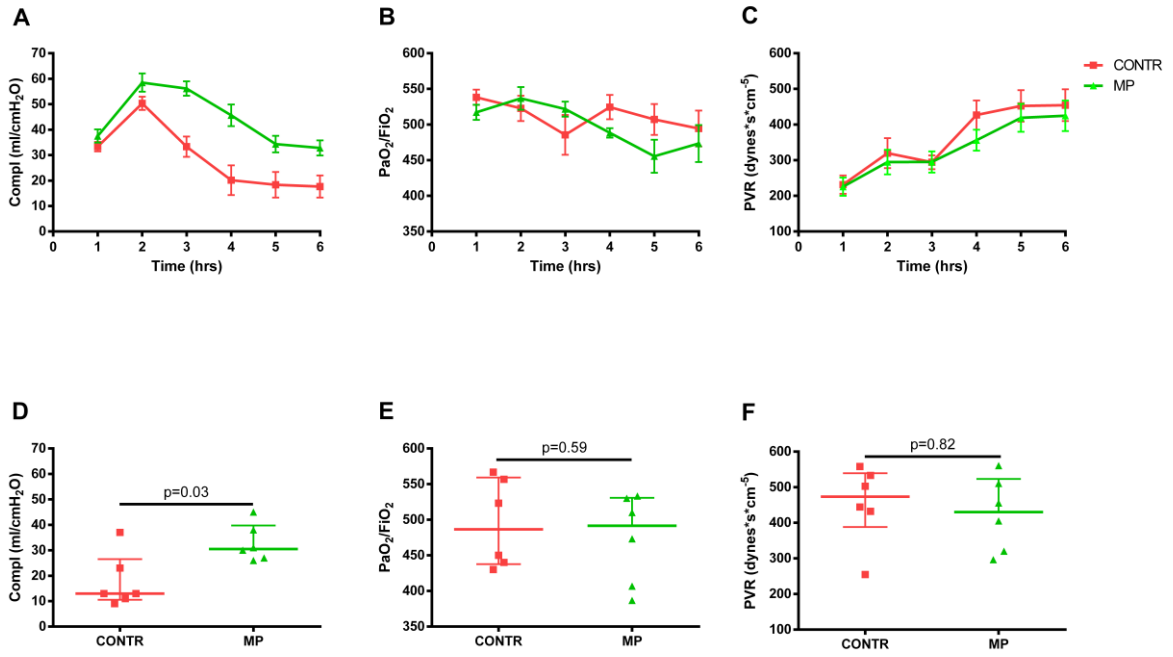

Figure III.1 – Dynamic airway compliance, oxygenation (PaO2/FiO2) and pulmonary vascular resistance are depicted during the 6 hours of ex-vivo lung perfusion in figure 1A, C, E respectively (median ± IQR) for both groups. Compliance, oxygenation and PVR are depicted at the end of EVLP (scatter plot median ± IQR) in figure 1 B, D, F respectively. A permutation test shows significantly better airway compliance at the end of EVLP in the MP-group (p=0.0304). All other parameters are not significantly different.

For all experiments in the MP-group, grafts could be perfused for 6 hours. In the CONTR-group however, there was a drop-out of 3 experiments where perfusion was ended on 3.75, 4.0 and 4.5 hrs respectively due to excessive edema formation (Figure III.2). The superior survival of the grafts in the MP-group nearly reaches significance (p=0.06).

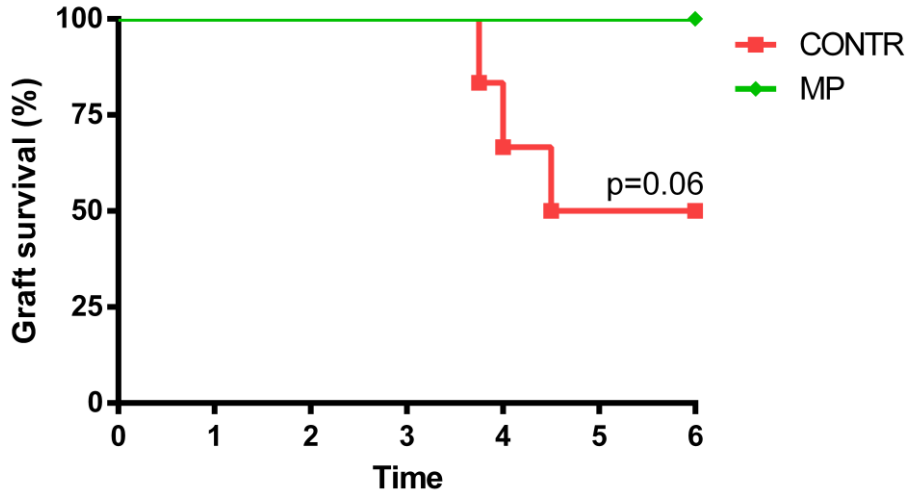

Figure III.2 – Survival of the lung graft on EVLP, perfusion was ended when 1000 ml of perfusate was transformed in lung edema and ventilation was impossible. There was a trend in better graft survival in the MP-group that only just failed to reach significance (log rank test p=0.06).

Assessment of pulmonary edema

A high W/D (median 6.9) is observed in the CONTR-group, and a low W/D (median 6.1) in the MP-group. W/D weight ratio is significantly lower (p=0.02) in the group that received methylprednisolone (Figure III.3A). Density measurements on CT-scan confirmed this excess of extravascular lung water accumulation and showed a significantly higher (p=0.002) density in the CONTR-group (median 353 g/L) compared to the MP-group (median 183 g/L) (Figure III.3B). Septal thickening and severe lung edema can clearly be visualized in the CONTR-group (Figure III.3C) in comparison to the MP-group (Figure III.3D).

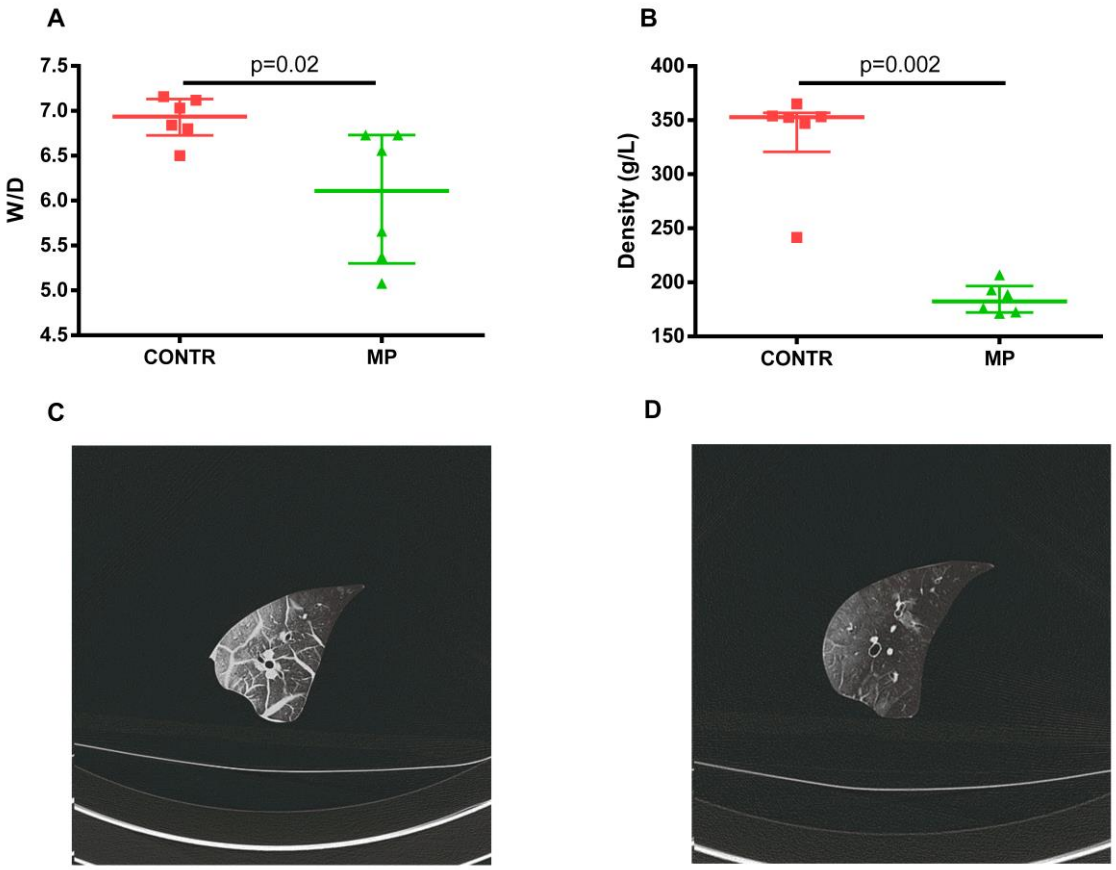

Figure III.3 – A) W/D is depicted as a scatter plot (median ± IQR). A permutation test of the W/D shows significantly less lung edema in the MP-group (p=0.02). B) CT-scan density is depicted as a scatter plot (median ± IQR). A permutation test shows a significantly higher CT-density in the CONTR-group compared to the MP-group (p=0.002). C) CT-scan of left lower lobe in CONTR-group. D) CT-scan of left lower lobe in MP-group.

Histology

Histological analysis of lungs in the MP-group did not reveal any significant differences in congestion (p=0.58), neutrophil invasion (p=0.13) and membrane disruption (p=0.21) compared to the CONTR-group (Figure III.4).

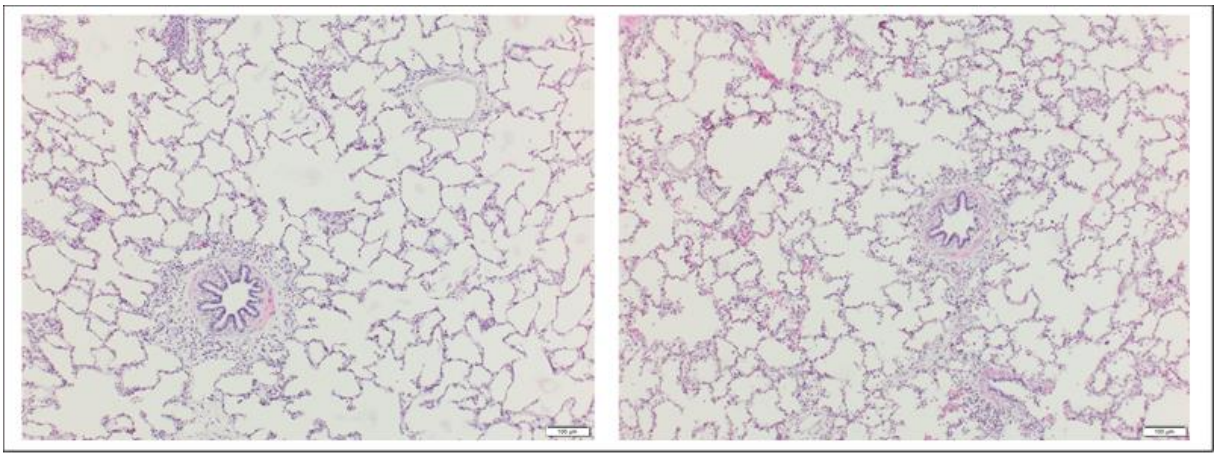

Figure III.4 – Similar histological findings in CONTR-group (left) as in MP-group (right)

Immunological evaluation

Porcine multiplex analysis of the perfusate sample (Figure III.5) at the end of EVLP showed a median IL-1β level of 124.6 pg/ml in the CONTR-group vs 39.8 pg/ml in the MP-group (p=0.002); a median IFN-α level of 94.6 pg/ml in the CONTR-group vs 0.7 pg/ml in the MP-group (p=0.004); a median TNF-α level of 3181 pg/ml in the CONTR-group vs 226 pg/ml in the MP-group (p=0.008); a median IL-10 level of 94.6 pg/ml in the CONTR-group vs 0.7 pg/ml in the MP-group (0.004) and a median IFN-γ level of 2.8 pg/ml in the CONTR-group vs 0.08 pg/ml in the MP-group (p=0.005). IL-8 was above detection limit in the CONTR-group (highest standard depicted), but low in the MP-group (median 45.3 pg/ml). IL-4 was below the detection limit.

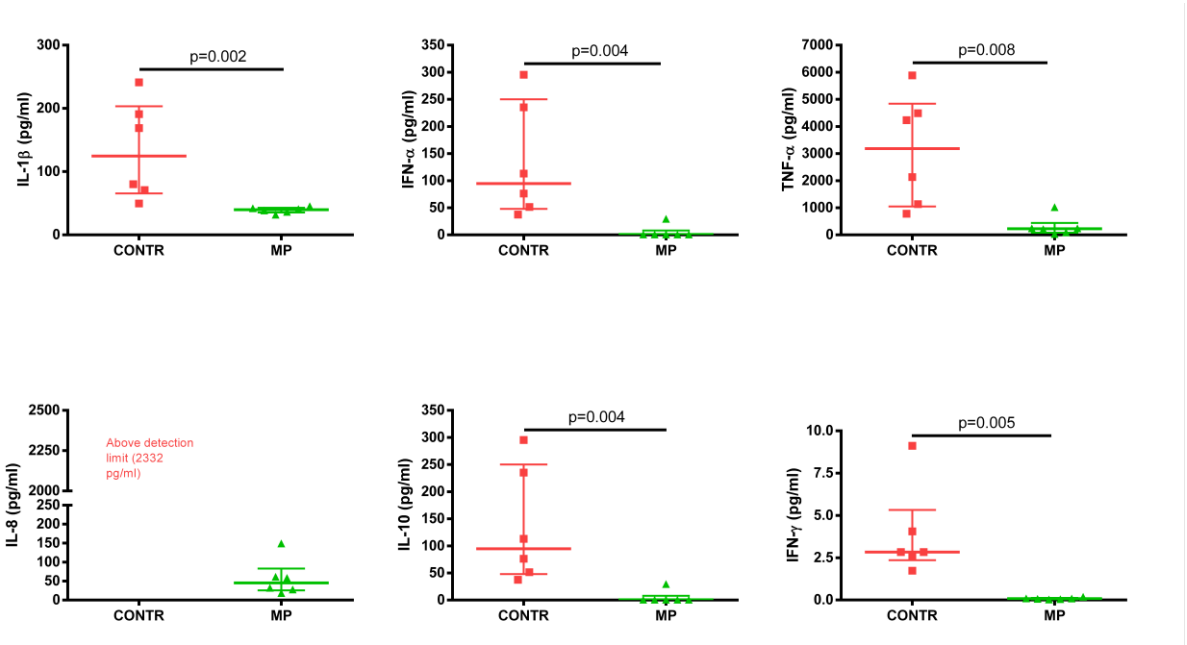

Figure III.5 – Porcine multiplex analysis of the perfusate samples at the end of EVLP. IL-1β, IFN-α, TNF-α, IL-10 and IFN-γ are all significantly lower in the MP-group. IL-8 was above detection limit in the CONTR-group, but low in the MP-group. IL-4 was below detection limit and is not depicted. Data points are depicted in a scatter plot with median ± IQR and the resulting p-value of the permutation test.

Multiplex analysis of the BAL fluid at the end of EVLP showed a median IL-1β level of 36.5 pg/ml in the CONTR-group vs 34.2 pg/ml in the MP-group (p=0.29); a median IFN-α level of 0.36 pg/ml in the CONTR-group vs 0.38 pg/ml in the MP-group (p=0.56); a median TNF-α level of 234 pg/ml in the CONTR-group vs 105 pg/ml in the MP-group (p=0.17) and a median IL-8 level of 174.2 pg/ml in the CONTR-group vs 61.8 pg/ml in the MP-group (p=0.06). IFN-γ was significantly lower (p=0.02) in the MP-group (median 0.05 pg/ml) compared to the CONTR-group (median 0.1 pg/ml). IL-10 and IL-4 were both below the detection limit.

F) DISCUSSION

We report our experimental findings on the role of steroids in a DCD model of organ donation. To our knowledge, this is the first experimental report on the use of steroids in a DCD donor. We demonstrated that administration of steroids prior to warm ischemia and during EVLP evaluation significantly improved lung function, lung edema and reduced a subset of inflammatory markers.

By giving steroids to the donor prior to the onset of warm ischemia and further exposure to steroids during EVLP, we observed improved lung compliance at the end of EVLP. Pulmonary vascular resistance and oxygenation did not differ between both groups. However, it has previously been advocated that compliance is the best parameter to predict donor lung quality (28,29) since this parameter directly reflects the impact of fluid extravasation in the lung. Also, physiological acceptance criteria for transplantation after EVLP are not yet been agreed upon, and other groups do advocate the use of oxygenation and pulmonary vascular resistance as the best evaluation parameters with excellent results after transplantation (30). W/D is still the golden standard for estimation of lung edema, and in our experimental study it was significantly lower in the MP-group. This could be further validated by a lower density measurement in the methylprednisolone group on CT-scan. The latter provides assessment of the whole lung surface, while a biopsy provides information only on a small portion of the tissue. Implementation of CT-scanning might be considered as a valuable non-invasive tool to measure pulmonary edema.

The cytokine expression profile of lungs in both groups was represented by evaluating cytokines in both the circulating perfusate and BAL (at the end of EVLP). Administration of steroids to the donor in addition with exposure to steroids during EVLP resulted in a decreased level of cytokine production and release, especially in the perfusate. Also, this reflects a reduced organ inflammation, but the role of cytokine expression on EVLP is still largely unknown (31).

It might be that a different pattern of cytokines is expressed during ex vivo organ perfusion that does not completely reflect the in vivo reperfusion situation. We used an acellular perfusate and the reperfusion injury observed during our set-up is mainly driven by resident leukocytes in the pulmonary graft. This expressed cytokine panel also suggest an important role for macrophage secretion. The observation that the anti-inflammatory cytokine IL-10 was also significantly reduced indicates that we should better look at the balance between pro- and anti-inflammatory mediators, rather than the absolute concentration.

Early outcome after lung transplantation is mainly impaired by the occurrence of severe primary graft dysfunction (PGD) driven by ischemia-reperfusion injury and occurs in up to 30% of lung transplant recipients (32,33). Despite better supportive treatment options such as extra-corporeal membrane oxygenation (34) to limit early mortality from severe PGD, this syndrome has a significant impact on long-term outcome with an increased 90-day and 1-year mortality after severe PGD at 72 hrs after lung transplantation (33). Also, there is an increased risk to develop bronchiolitis obliterans syndrome (BOS) (32,35–37) following high-grade primary graft dysfunction.

In addition, the use of DCD organs itself seems to be an increased risk factor for PGD. Although similar short- and long-term outcomes between DBD and DCD donors have been reported (38–40). Therefore, it is of great interest to limit primary graft dysfunction after lung transplantation with a specific strategy such as steroid administration. The possible benefit of steroid administration is already been highlighted in brain-dead organ donation (25,41). That is, steroids can suppress the cytokine release during the catecholamine storm and improve hemodynamic stability in adrenal insufficient patients (25,42). Controlled DCD donors suffer an agonal phase that is unpredictable, prior to circulatory arrest. This agonal phase can add a large variability to the injury and is difficult to standardize. In a previous study (43) we have investigated the impact of different modes of death in DCD donation. We could identify that

hypoxic arrest was more detrimental to the graft quality (44). In our current study, we have chosen to work with a standardized warm-ischemic porcine DCD model, with immediate onset of the warm-ischemic interval by induction of ventricular fibrillation and disconnection of the ventilator (in a paralyzed animal). In this way, we could better standardize the warm-ischemic injury (still the most important component of IRI in DCD donation). The effect of steroids in a controlled DCD model induced by hypoxic arrest with variable periods of warm ischemia is also an interesting study to conduct in the future.

The major limitation of this study is the absence of a control group where steroids are used only in the donor animal. However, steroids are included in all EVLP protocols without convincing evidence for a beneficial effect on PGD. Based on previous preliminary data in our laboratory we are confident that steroid administration post-injury during EVLP only, cannot reverse warm-ischemic injury. This is also shown from other research experiments where steroids are applied in the perfusate in both control and treatment groups (45–47). Therefore, we believe that it is the administration of steroids to the DCD donor prior to circulatory arrest that is important for optimal organ preservation to alleviate warm-ischemic damage. The administration window of preconditioning and preservation strategies is still largely unknown. In order to avoid missing any positive effect by focusing on a narrow window, we chose to expose the grafts to steroids throughout the whole experiment. Of course, further experiments should now be designed to elucidate the role of the pre-arrest donor treatment or treatment of the graft during EVLP only. Also, our findings need to be validated in a transplant model.

In case of donation after brain death (DBD), the advantage of using steroids has been investigated previously (25). However, the role of steroids in DCD donation has never been investigated. The reason is two-fold: firstly, DCD donation has only recently become of higher interest and research in donor management of the DCD donor is limited and difficult to design. Secondly, the dead donor rule impedes on any intervention in the patient awaiting therapy

withdrawal (controlled DCD donor) (48). However, harmless therapies such as the use of heparin have been widely adopted in various European countries to be used in DCD protocols (3,10). A relation between the administration of heparin and the acceleration of the dying process has not been demonstrated so far. We do not know if there is a relation between the dying process or length of the agonal phase and the administration of high dose glucocorticoids. This opens up the discussion about expanding donor management to DCD, as it is more and more applied in DBD programs. Since steroids are not harmful to patients, we believe that steroid administration to DCD donors should be considered. Despite this ethical issue, the latest report of the DCD-registry within the International Society of Heart and Lung Transplantation noted that already over 90% of the participating centers give steroids to the DCD donor prior to declaration of dead (3). Unfortunately, there is no data available on the doses, frequency and timing of steroid administration in the DCD donation process. We therefore do not know whether steroids were administered during the ICU admission as a treatment to reduce cerebral edema, or whether it was administered to optimize donor organ quality. If these steroids would have been administered intentionally to optimize donor quality, this course of action coincides with the dead-donor rule since therapy is given to a patient who is not declared dead yet. Some believe that this policy could bring harm to transplant programs. However, others believe that once the decision for switch-off and organ donation has been made, one can go forward with donor management and optimization protocols which should be performed by an independent team to avoid any conflict of interest or harm to the donor. We believe that our findings should further be embedded in an ethical discussion to decide if pre-treatment in a DCD donor is ethically and legally acceptable. Donor management of a DCD donor would however be most feasible with interventions that are beneficial when applied only just prior to circulatory arrest (as shown in these experiments). This avoids implementation of complex and time-consuming management protocols prior to the controlled DCD procedure.

We conclude that administration of steroids to a DCD donor and during ex-vivo lung perfusion attenuates warm ischemia-reperfusion injury. The role of steroids during ex-vivo lung perfusion only should be the subject of future research. In addition, a study on the effect of steroids administered only to the donor, in a controlled DCD model with hypoxic arrest will also add knowledge in the future. We advocate the use of steroids in clinical DCD programs worldwide, with caution to further introduce preconditioning strategies prior to declaration of death in organ donation programs.

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CHAPTER IV
EVLP POTENTIAL AND FEASIBILITY

IV.A A SINGLE-CENTER ANALYSIS OF REJECTED DONOR LUNGS AND THE CLINICAL POTENTIAL OF EVLP

Submitted for publication:
Martens A, Van Raemdonck D, Smits J, Verleden SE, Vos R, Vanaudenaerde BM, Verleden GM, Degezelle K, Desschans B, Neyrinck AP. A retrospective database analysis to evaluate the potential of EVLP to recruit declined lung donors

A) PREFACE

In the previous chapter (chapter III), we showed that advancements during the donor phase can increase both number and quality of transplantable donor lungs. In this chapter, we will focus on optimization of the preservation phase by investigating the actual potential and clinical implementation of ex-vivo lung perfusion (EVLP). EVLP is a normothermic preservation strategy, used as an alternative to cold static preservation. It allows for additional evaluation, optimal preservation and even improvement of lung quality prior to transplantation. Therefore, it is introduced as the ideal method to increase both number and quality of donor lungs. However, the true impact of EVLP is still largely unknown. In this chapter, we report on our analysis of registered donor data of donors whose lungs were not transplanted, to provide insights in which lungs can potentially be recovered by EVLP to increase number and quality of transplantable donor lungs.

B) ABSTRACT

Background - Ex-vivo lung perfusion (EVLP) is already being used for both standard and extended-criteria donor (ECD) lungs. For further expansion of the transplant activity, we might have to extend the threshold for ECD donation. The purpose of this study was to estimate how many of those ECD lungs could be recruited by EVLP.

Methods - We reviewed all multi-organ donors (MODs) from our collaborative donor hospitals (January 2010 - June 2015). All unused lung donors, were categorized using registered donor data and evaluated by two independent investigators to identify which lungs could be transplanted after EVLP.

Results - 584 MODs were registered at our transplant center within our collaborative donor hospitals network. 268 (45.9%) were declined as lung donor at the moment of registration ("declined as lung donor") and 316 (54.1%) were considered as a donor for lung transplantation ("considered as lung donor"). In the latter, lungs from 220 (37.7%) donors were transplanted and 96 donors (16.4%) were not. Donors whose lungs were not transplanted (declined and considered) were categorized as: no consent; donor-related factors; death-related organ injury; logistical reason; unknown. Across the different groups, we identified 78 out of 364 declined donors (21.4%) whose lungs could benefit from EVLP; and potentially become suitable for lung transplantation.

Conclusions – With this retrospective database analysis of unused lung donors, we identified a large potential for EVLP to further increase the donor pool in transplant centers where the majority of donor lungs are already extended.

C) INTRODUCTION

Lung transplantation has become a successful treatment strategy for an increasing amount of well-selected patients with end-stage pulmonary diseases, due to surgical improvements and the development and optimization of immunosuppressive therapy. Unfortunately, only a small proportion of multi-organ donors (MODs) are currently suitable to donate lungs for transplantation. Reported acceptance rates vary from 15 to 35% between centers (1,2). Consequently, the patients on the waiting list outnumber the amount of transplantable organs, resulting in a persistent waitlist mortality (3).

Therefore, many strategies have been explored to increase the availability of transplantable donor lungs to improve outcome for patients in need of a pulmonary allograft (4,5).

First, we are increasingly transplanting donor organs that do not fulfill the strict criteria of lung transplantation, the so-called extended-criteria donors (ECD) to enlarge the existing pool of standard-criteria donors (SCD) (6). Secondly, ex-vivo lung perfusion (EVLP) was introduced in the lung transplant field by Stig Steen in 2001, as a tool to expand the current donor pool by re-evaluating donor lungs prior to transplantation (7). EVLP is a normothermic perfusion technique that can be achieved by a pump-driven perfusion machine which circulates a preservation solution through the vasculature of the lung. Lungs are also ventilated ex-vivo and can be continuously monitored and evaluated. Therefore, it has been proposed as the ideal method to recruit more donor organs as we can evaluate questionable donor organs and potentially even increase their quality. Recently we became interested in not only re-assessing the donor organ on EVLP, but also preserving it in superior circumstances compared to static cold preservation.

Several research groups have already published their initial experience with perfusing high-risk or extended-criteria donor lungs with EVLP prior to transplantation to expand the donor pool (8,9). However, although some groups classify these ECD donor lungs as “initially rejected

donor lungs" (10,11), we believe that EVLP is not always mandatory to safely transplant ECD lungs. Both short-term and long-term outcome after ECD lung transplantation without EVLP has been reported to be comparable with SCD donor lung transplantation provided that they are allocated to a suitable patient with an acceptable survival probability (12,13). Also in our center, ECD lung transplantation results in comparable long-term outcome compared to SCD lung transplantation (6,14).

If we want to further implement EVLP in clinical practice, it is crucial to estimate the potential of EVLP to increase transplant activity and donor organ quality. Therefore, the aim of this study was to retrospectively review our database of multi-organ donors and to analyze the reasons of donor lung decline and the conditions where lungs could be salvaged by EVLP technology.

D) METHODS

Data collection and categorization

We retrospectively reviewed our database of all MODs offered to our center from January 2010 until June 2015. Our transplant center is organized within a network of 33 collaborative donor hospitals and we report all offers within this network to Eurotransplant.

First, we divided all MODs in two categories: "*declined as lung donor*" and "*considered as lung donor*". The decision to consider a MOD as a potential lung donor, was driven by the expert opinion of our transplant physicians that is based on interpretation of both medical and technical information provided by the donor center. MODs considered to be good candidates to donate lungs for transplantation were allocated by Eurotransplant, based on international allocation rules (15). The actual number of donors whose lungs were finally accepted for transplantation were recorded, these lungs could be allocated by Eurotransplant first to other centers or were transplanted in our center based on our local allocation system. They were categorized as ECDs if one or more of the following criteria was met: age>55, $PaO_2/FiO_2<300$, abnormal chest x-ray, smoking history, presence of aspiration, presence of chest trauma or donation after circulatory death (DCD). All other lungs were considered SCDs.

Secondly, we categorized all MODs whose lungs were not transplanted. This group included the lungs that were initially declined as lung donor ("*declined as potential lung donor*"), the grafts that were declined after receiving additional information from the donor center prior to leave for procurement ("*declined without in situ evaluation*") and the grafts that were ultimately declined in situ after opening the chest with direct macroscopic assessment of the lungs ("*declined after in situ evaluation*").

Next, data of donors whose lungs were not transplanted were re-assessed individually by two independent investigators to identify the reason for decline. This assessment was done using the available donor data within the database and based on consensus between the investigators.

With this information, all non-transplanted donor lungs were assigned to subcategories listed in Table IV.1. If more than one reason was identified, the most important factor (as assessed by the investigators) was listed.

Table IV.1 - Subcategories of lung donors declined for transplantation

No consent	Logistical reason	Donor-related factors	Death-related organ injury	Unknown
Family Refusal	No matched recipient	Advanced age	Abnormal arterial blood gases (low PaO_2/FiO_2)	No information available on why lungs were rejected
No consent medical examiner	Procurement team not present in time	Smoking	Abnormal chest X-ray/CT-scan	No reason found based on donor data, technical investigations or blood gases
	No operating room or surgical team available	Chronic obstructive pulmonary disease (COPD)	Aspiration	
		Malignancy	Hemodynamic unstable donor	
		Systemic disease	Pulmonary emboli	
		Pulmonary fibrosis	Lung edema	
		Pleural disease	Parenchymal haematoma/ contusion/ polytrauma	
			Pulmonary infection/Systemic infection	
			Unknown warm-ischemic time (DCDII)	

EVLP candidate selection

Finally, two investigators (A.M. & A.P.N) identified potential donor grafts among the non-transplanted organs that could be salvaged by using EVLP technology. The manner of recovering these organs was based on reassessment (additional evaluation of the organ function), improved preservation (prolonged out-of-body time) and reconditioning (potential improvement of specific injuries during EVLP). The selection criteria for currently rejected donor lungs that potentially could be salvaged by EVLP were: neurogenic lung edema,

pulmonary emboli, PaO_2/FiO_2 below 300 without obvious explanation, minor pulmonary infections (consolidation on chest x-ray or purulent sputa with PaO_2/FiO_2 above 200), unknown warm-ischemic time and logistical reasons for donor lung decline (extended time for allocation or to schedule the transplant procedure) (Table IV.2).

Table IV.2 – Selection criteria for rejected donor lungs to be recovered by EVLP

Selection criteria EVLP recovery:
-Neurogenic lung edema
-Pulmonary emboli
-PaO_2/FiO_2 < 300 without obvious explanation
-Minor pulmonary infection (consolidation on chest x-ray or purulent sputa with PaO_2/FiO_2 > 200)
-Unknown warm ischemic time
-Logistical reasons

PaO_2 / FiO_2 = Partial oxygen pressure / fractional inspired oxygen concentration

E) RESULTS

Categorization of MOD offers

584 MODs were recorded at our center between January 2010 and June 2015 (= *"multi-organ donors"*).

Of those 584 MODs registered within our collaborative donor hospitals, 268 (45.9%) were declined as lung donor and lungs were not allocated (= *"declined as potential lung donor"*). 316 (54.1%) MODs were considered as a lung donor (= *"considered as potential lung donor"*) and were reported to Eurotransplant for allocation. However, 53 (9%) were declined based on additional information or second evaluation of donor data by the transplant center to which lungs were allocated (= *"declined without in situ evaluation"*). Another 43 (7.4%) were declined upon procurement in the donor hospital (= *"declined after in situ evaluation"*).

Lungs from 220 MODs were successfully transplanted (= *"transplanted"*). Of those, 72% could be categorized as ECDs based on previously published criteria (6), and only 28% were SCDs. Donor characteristics of the individual categories are listed in Table IV.3.

Declined lung donors

In the *"declined as potential lung donor"* group, 3 MODs were declined since there was no consent for organ donation. Donor-related factors (n=106) included: old age (n=58), a history of smoking or COPD (n=35), or a significant medical history (n=13) such as pulmonary hypertension, cardiovascular disease and malignancy. Death-related organ injury (n=150) included: abnormal chest X-ray/arterial blood gases/bronchoscopy results (n=71), pulmonary infection (n=32), aspiration (n=19), poly-trauma with lung contusion (n=14), DCD category II (n=7), a hemodynamic instable donor with unknown warm ischemic time (n=4), presence of pulmonary emboli (n=2), or neurogenic lung edema (n=1). In 9 cases, no obvious reason could be identified why lungs were not transplanted based on the registered donor data and medical investigations.

Table IV.3 – Descriptive analysis of all MODs and their subgroups

	MODs n=584	Declined as potential lung donor n=268	Considered as potential lung donor n=316	Declined as lung donor without in situ evaluation n=53	Declined as lung donor after in situ evaluation n=43	Donors used for lung transplantation n=220
		NOT ALLOCATED	ALLOCATED			
Gender						
Male	327 (56%)	149 (55.6%)	178 (56.3%)	34 (64.2%)	30 (69.8%)	1114 (51.8%)
Female	247 (42.3%)	109 (40.7%)	138 (43.7%)	19 (35.8%)	13 (30.2%)	106 (48.2%)
Not registered	10 (1.7%)	10 (3.7%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Donor age (y)						
Median (25% IQR - 75% IQR)	*56 (45 - 66)*	*62.0 (49 - 76)*	*52 (43 - 62)*	*55 (41 - 65)*	*50 (44 - 61)*	*52 (43.5 - 61.0)*
<45	143 (24.5%)	52 (19.4%)	91 (28.8%)	17 (32.1%)	12 (27.9%)	62 (28.2%)
45-54	125 (21.4%)	34 (12.7%)	91 (28.8%)	9 (17.0%)	12 (27.9%)	70 (31.8%)
55-59	65 (11.1%)	28 (10.5%)	37 (11.7%)	7 (13.2%)	4 (9.3%)	26 (11.8%)
>60	241 (41.3%)	144 (53.7%)	97 (30.7%)	20 (37.7%)	15 (34.9%)	62 (28.2%)
Not registered	10 (1.7%)	10 (3.7%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
PaO2/FiO2						
Median (25% IQR - 75% IQR)	*393 (298 - 478)*	*296.0 (188 - 402)*	*436 (371 - 503)*	*392 (331 - 469)*	*414 (366 - 489)*	*449 (383 - 508)*
>450	170 (29.1%)	34 (12.7%)	136 (43.1%)	15 (28.3%)	14 (32.6%)	107 (48.6%)
351-450	172 (29.4%)	49 (18.3%)	123 (38.9%)	20 (37.7%)	21 (48.8%)	82 (37.3%)
301-350	50 (8.6%)	18 (6.7%)	32 (10.1%)	8 (15.1%)	5 (11.6%)	19 (8.6%)
≤300	122 (20.9%)	103 (38.4)	19 (6.0%)	10 (18.9%)	2 (4.7%)	7 (3.2%)
Not registered	70 (12.0%)	64 (23.9%)	6 (1.9%)	0 (0%)	1 (2.3%)	5 (2.3%)
Smoker						
Yes	196 (33.5%)	86 (32.1%)	110 (34.8%)	21 (39.6%)	21 (48.8%)	68 (30.9%)
No	309 (52.9%)	139 (51,9%)	170 (53.8%)	27 (51.0%)	18 (41.9%)	125 (56.8%)
Not registered	79 (13.5%)	43 (16.0%)	36 (11.4%)	5 (9.4%)	4 (9.3%)	27 (12.3%)
Donor category						
DBD	463 (79.3%)	209 (78.0%)	254 (80.4%)	39 (73.6%)	32 (74.4%)	183 (83.2%)
DCD	121 (20.7%)	59 (22.0%)	62 (19.6%)	14 (26.4%)	11 (25.6%)	37 (16.8%)
BMI						
Median (25% IQR - 75% IQR)	*24.7 (22.5 - 27.7)*	*25.3 (23.2 - 27.8)*	*24.6 (22.1 - 27.3)*	*26.2 (22.8 - 28.6)*	*25.3 (22.5 - 27.8)*	*24.3 (22.0 - 26.7)*
<20	46 (7.9%)	21 (7.9%)	25 (7.9%)	7 (13.2%)	4 (9.3%)	14 (6.4%)
20-25	255 (43.6%)	100 (37.3%)	155 (49.1%)	15 (28.3%)	17 (39.5%)	123 (55.9%)
>25	272 (46.6%)	137 (51.1%)	136 (43.0%)	31 (58.5%)	22 (51.2%)	83 (37.7%)
Not registered	11 (1.9%)	10 (3.7%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Chest X-ray						
Clear	215 (36.8%)	53 (19.8%)	162 (51.3%)	21 (39.6%)	23 (53.5%)	118 (53.6%)
Consolidation	168 (28.8%)	92 (34.3%)	76 (24.0%)	15 (28.3%)	10 (23.2%)	51 (23.2%)
Atelectasis	48 (8.2%)	24 (8.9%)	24 (7.6%)	6 (11.3%)	3 (7.0%)	15 (6.8%)
Pleural Fluid	14 (2.4%)	9 (3.3%)	5 (1.6%)	1 (1.9%)	3 (7.0%)	1 (0.5%)
Contusion/pneumothorax	11 (1.9%)	4 (1.5%)	7 (2.2%)	3 (5.7%)	0 (0%)	4 (1.8%)
Edema	14 (2.4%)	3 (1.2%)	11 (3.5%)	2 (3.8%)	1 (2.3%)	8 (3.6%)
Not registered	114 (19.5%)	83 (31.0%)	31 (9.8%)	5 (9.4%)	3 (7.0%)	23 (10.5%)
Bronchoscopy						
Clear	7 (1.2%)	0 (0%)	7 (2.2%)	1 (1.9%)	0 (0%)	6 (2.7%)
Non-purulent secretions	17 (2.9%)	2 (0.7%)	15 (4.8%)	3 (5.7%)	2 (4.7%)	10 (4.5%)
Purulent secretions	19 (3.3%)	8 (3.0%)	11 (3.5%)	4 (7.5%)	2 (4.7%)	5 (2.3%)
Inflammation	2 (0.3%)	0 (0%)	2 (0.6%)	0 (0%)	0 (0%)	2 (0.9%)
Not registered	539 (92.3%)	258 (96.3%)	281 (88.9%)	45 (84.9%)	39 (90.6%)	197 (89.6%)
Lung Donor Score (ET)						
Median (25% IQR - 75 IQR)	*8 (7 - 9)*	*9 (8 - 10)*	*8 (7 - 8)*	*8 (7 - 10)*	*8 (7 - 9)*	*7 (7 - 8)*
≤7	175 (30.0%)	29 (10.8%)	146 (46.2%)	16 (30.2%)	17 (39.5%)	113 (51.4%)
8	158 (27.0%)	54 (20.1%)	104 (32.9%)	14 (26.4%)	15 (34.9%)	75 (34.1%)
9	119 (20.4%)	78 (29.1%)	41 (12.9%)	9 (17.0%)	9 (20.9%)	24 (10.9%)
≥10	132 (22.6%)	107 (40.0%)	25 (8.0%)	14 (26.4%)	2 (4.7%)	8 (3.6%)

Lung donor score determined by Eurotransplant (ET) criteria (13)

In the "*declined without in situ evaluation*" group (n=53), no suitable recipient could be found in time within the Eurotransplant database in 22 cases. The lung procurement team did not arrive on time in 4 cases where the abdominal procurement team already started to avoid long

warm ischemic times in a hemodynamically instable donor. Donor-related factors (n=13) included: a significant medical history that interfered with transplantability (n=10), a severe smoking history that was not previously reported (n=2), or high donor age (n=1). Death-related organ injuries (n=11) referred to an abnormal chest x-ray in 5 cases, to pulmonary infection in 3 cases, or abnormal arterial blood gases in 3 cases. In another 3 cases, no obvious reason could be identified why lungs were not transplanted based on the registered donor data and medical investigations.

In the "*lungs declined after in situ evaluation*" group (n=43), donor-related factors (n=16) leading to decline of the lung donor for transplantation included: intrinsic lung diseases such as emphysema (n=14), fibrosis (n=1) and pleural disease (n=1). Death-related organ injuries (n=21) that led to inability to transplant the donor lungs were identified as: a significant pulmonary infection (n=10), abnormal arterial blood gases (n=6), pulmonary emboli (n=2), parenchymal hematoma (n=2), or severe lung edema (n=1). In 6 cases, no obvious reason could be identified why lungs were not transplanted based on the registered donor data and medical investigations.

All categories and subcategories, including the lung donors that were selected as candidates for EVLP-recovery are summarized in Figure IV.1.

Candidates for ex-vivo lung perfusion

In total, lungs of 78 lung donors were identified as potential candidates to be recovered by EVLP evaluation, preservation and reconditioning based on expert opinion. From the group "*declined as potential lung donor*", these included: 8 MODs with minor pulmonary infection, 7 DCD II donors, 7 MODs with low arterial blood gases without obvious reason, 5 with atelectasis, 4 hemodynamically unstable MODs with unknown warm ischemic time, 3 MODs with neurogenic lung edema, and 1 MOD with lung emboli.

In the "*declined without in situ evaluation*" group, cases in which a logistical reason led to refusal of the lung donor (n=26) and MODs with minor pulmonary infections (n=5) were considered as good candidates for EVLP evaluation-preservation-reconditioning.

In the "*declined after in situ evaluation*" group, lungs of potential lung donors could potentially be recovered if they would have been carefully evaluated or actively reconditioned on EVLP in 6 cases of minor infection, abnormal arterial blood gases (n=3), lung edema (n=1), or macroscopic appearance of lung emboli (n=2).

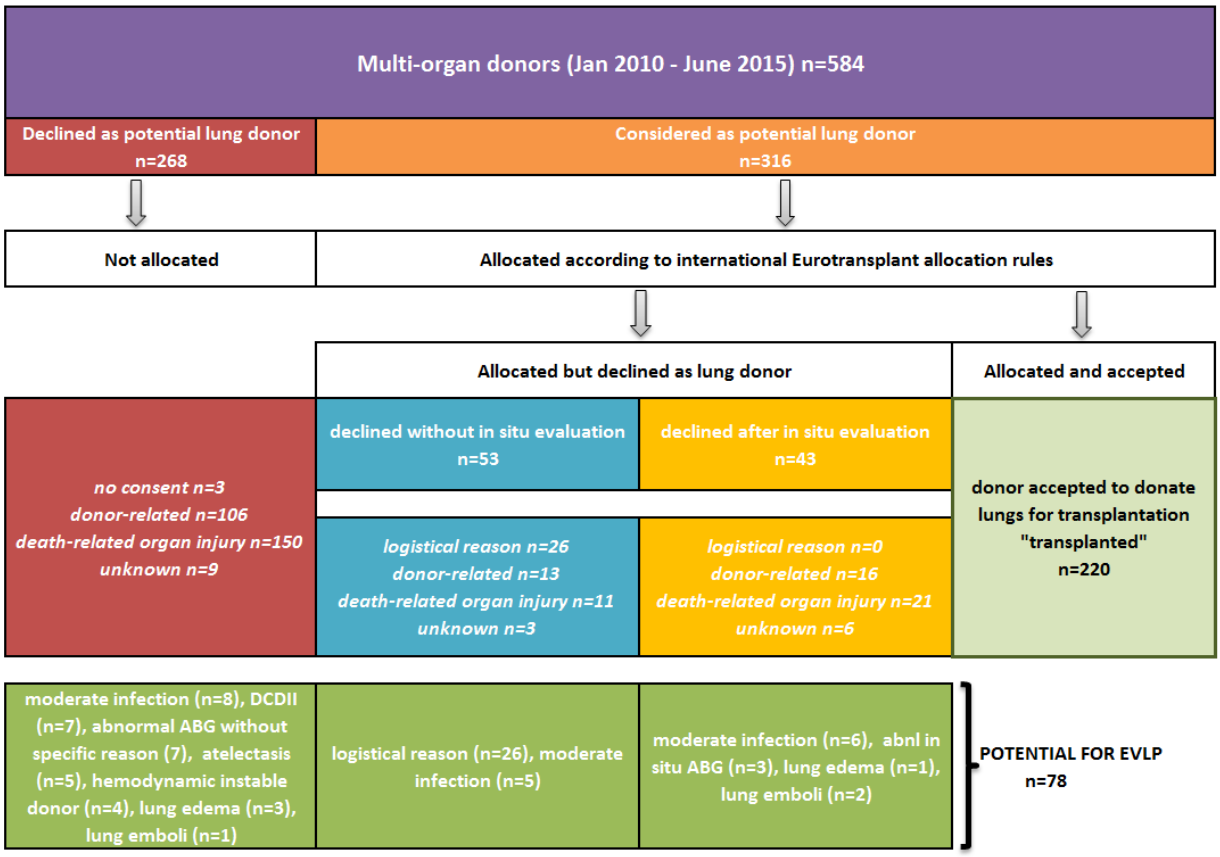


Figure IV.1 - Overview of multi-organ donors (purple) that were further categorized as "declined as potential lung donor" (red) or as "considered as potential lung donor" (orange). Also in the latter, they could still be declined without in situ evaluation (blue) or after in situ evaluation (yellow). Lungs of 220 out of 584 donors were actually transplanted. Lungs that were not transplanted, but could be candidates for ex-vivo lung perfusion evaluation, superior preservation or active rehabilitation are listed in the green boxes below.

F) DISCUSSION

In our study, we retrospectively analyzed donor data registered in our center to provide insights in MODs that are declined as lung donor in current clinical practice. We hypothesized that EVLP could increase the donor pool when lungs would be more carefully evaluated, preserved in superior conditions or even be actively reconditioned.

In Belgium, deceased organ donation is based on presumed consent legislation (opting-out system) leading to a high rate of 29.1 deceased donations per million inhabitants (16). In this opting-out system every deceased individual is classified as a potential donor, in absence of an explicit opting-out for organ donation before death. In case family members object to organ donation of their relative, their wishes will be respected unless the patient is explicitly registered to be an organ donor (opting-in). In this database, organ donors that were not offered due to objection of the patient (opting-out) or family members were not registered because no consent was obtained (although legally this is not obligated because of the presumed consent legislation). In 2 cases, there was an unforeseen objection of the family during a second evaluation and the donor procedure was abandoned. In 1 particular case, the body was not released by the legal medical examiner after a suicide attempt, so we could not proceed to organ donation.

The acceptance rate of MODs for lung transplantation in our study population was 37.7%. 584 MODs were offered to our center by our own university hospital and a collaborative donor network of 33 local hospitals, over a period of 66 months (Jan 2010 – June 2015), which corresponds to an annual number of MODs of 88 (this does not include organs offered by ET out of our local donor network). On average in Belgium (2010-2015), 330 MODs are reported annually for transplantation to ET, of which 168 are considered as lung donor and 107 are actually used. This leads to an acceptance rate of 32.5% of all MODs for lung transplantation.

Overall in Eurotransplant, this percentage is even lower, between 2010-2015 the average acceptance rate of MODs for lung transplantation was 26.6% (3).

We believe that improved donor management is an important cornerstone that might explain this high acceptance rate of our MODs. Efforts to improve management strategies have been incorporated in Intensive Care Unit practice. These include protective ventilation strategies (17), fluid restriction, steroid administration and early identification of potential donors (18,19). Our high acceptance rate supports that optimal management strategies with preset goals should never be completely abandoned in favor of machine perfusion.

We defined a subset of criteria that could be used to select grafts that could be salvaged by EVLP. First, infection leads to a high number of rejected organs. Although it might not be feasible to completely resolve a pulmonary infection during only a limited perfusion time on EVLP, a reduction in the microbial load (20) and endotoxin levels has already been demonstrated and could increase the quality of the infected donor lung (20,21). Therefore, it seems feasible to transplant lungs that first seemed unfit because of pulmonary infections, since we can minimize the microbial load in those lungs with high dose antibiotic treatment. These lung grafts were also included as candidates for EVLP recovery. Secondly, EVLP could also provide a solution for many logistical issues since we can potentially prolong the preservation time of the donor organs before transplantation. This can be done by either placing the lungs on a portable EVLP device in the donor center (22), or alternatively, a stationary device after a longer cold ischemic time can be used (23). Which technique is superior is still a subject of debate. Thirdly, atelectasis could be reversed on ex-vivo lung perfusion by meticulous recruitment maneuvers without derecruitment by abdominal compression. Also, lungs with a low PaO_2 without any obvious reason (such as infection) could be recruited and evaluated carefully. Fourth, lungs with neurogenic lung edema could be dried out by perfusing the lungs with a perfusate high on oncotic pressure or by activation of the alveolar fluid clearance during

normothermic metabolism. Lastly, lungs with pulmonary emboli could also be salvaged by perfusion alone where small emboli can be washed out, or by the addition of fibrinolytics (24,25). In many cases, lungs are re-evaluated ex-vivo to guarantee a qualitatively good donor lung for transplantation. Unfortunately, not all lungs can be recovered by EVLP. For example, lungs that are injured by direct trauma are difficult to preserve on EVLP due to air leak and leakage of perfusate in the alveoli. Therefore, structural damage was considered as not salvageable.

Already in 2002, Ware et al estimated that 40% of lungs that were not suited for transplantation could be salvaged by more objective ex-vivo evaluation (26). Due to technical improvement and refinement of the technique, including the ability of longer perfusion time on EVLP, this percentage of organ recovery by EVLP could be higher as initially reported. The conversion rate of unused donor lungs to transplantable donor lungs with EVLP highly depends on the inclusion criteria for EVLP. Previous studies in experienced EVLP centers showed conversion rates of 55-95% with extended-criteria or high-risk donor lungs (8,9,20). Selection of EVLP candidates in this cohort among the rejected lungs remains a subjective process and goes beyond selecting extended-donor lungs such has been previously proposed. However, all donor data were independently evaluated by two EVLP experts who performed over 300 EVLP cases in clinical and pre-clinical setting.

Early outcome after lung transplantation with EVLP seems promising; however, the long-term outcome is not well characterized yet. Tikkanen et al showed a similar 1-, 3-, and 5-year graft survival, chronic lung allograft dysfunction (CLAD)-free survival and quality of life in their cohort of 63 EVLP-grafts. Freedom from CLAD was even superior in brain-dead donors when EVLP was used. Up to now, data on outcome after EVLP is limited to ECD and SCD donor lungs that are often transplanted in other donor centers without perfusion on EVLP prior to transplantation. Therefore, further expansion of inclusion criteria for EVLP reconditioning

should be validated in pre-clinical safety models by a thorough evaluation of these rejected donor lungs on EVLP. We are convinced that our analysis contributes to a better insight in selection of EVLP candidates and to the development of strategies to successfully implement this technology in daily transplant activity.

In this cohort, 72% of the used donors could be retrospectively categorized as ECD and were successfully transplanted. Our group has previously published similar long-term outcome for ECDs compared to SCDs in a cohort of 431 donors, from which 63% were ECDs (6).

The limitation of this study is its retrospective nature using registry data. Also, the categorization was done using expert opinion since there is limited evidence reported to guide this analysis. However, this study did result in an improved donor database registration in our transplant center. Donor lungs that are not transplanted are now currently assigned to categories 1-4 based on the medical history of the donor, acute donor or organ injury, logistical reasons, or technical investigations that led to organ decline. Also, the timing of refusal of the donor lungs will be registered for each declined lung donor. Figure IV.2 gives an overview of the set-up of our current donor data registration system, which we recently implemented following the limitations we faced with the retrospective database analysis. These data can be used in the future for better prospective analysis of our registered donor data.

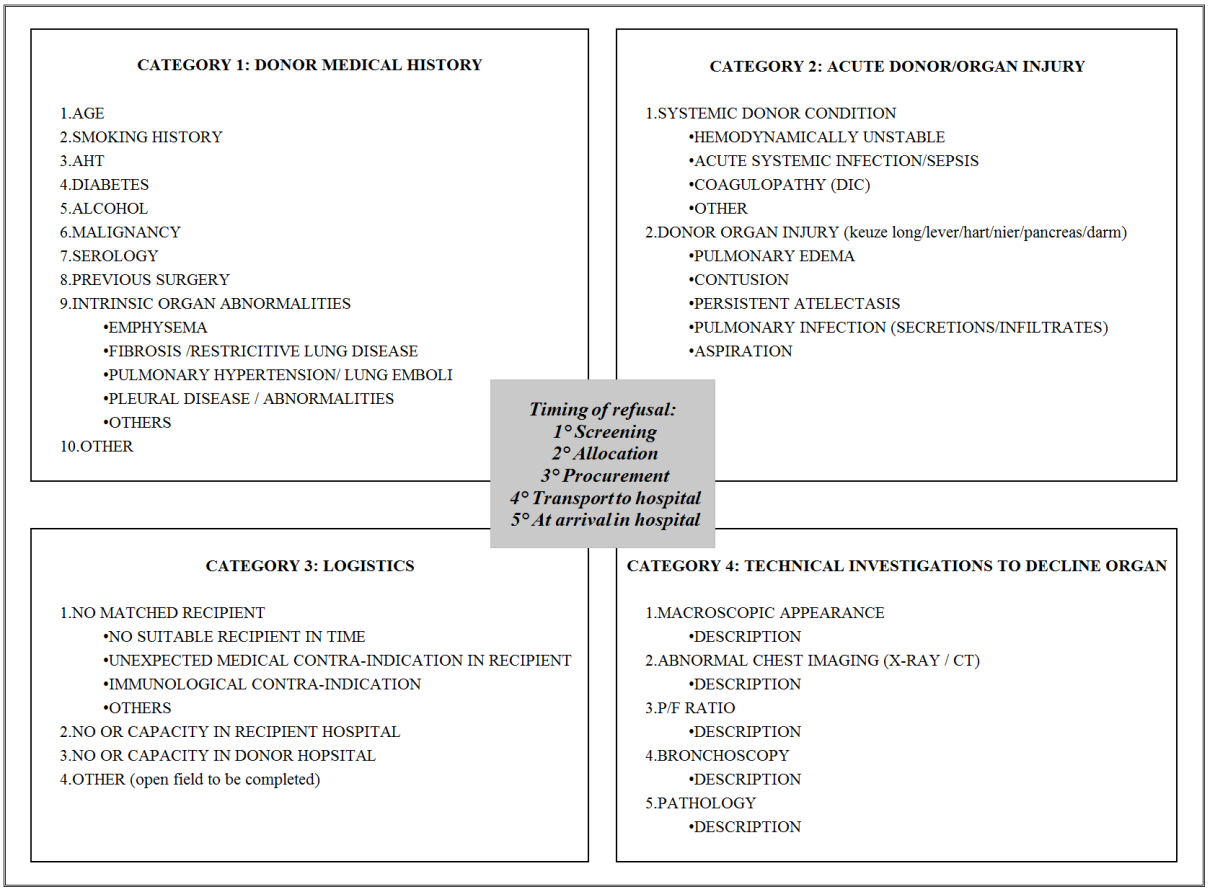


Figure IV.2 – Donor lungs that are not transplanted will be assigned to category 1 to 4 based on the medical history of the donor, acute donor or organ injury, logistical reasons or technical investigations that led to organ decline. Also, the timing of refusal of the donor lungs will be registered for each declined donor lung.

In addition, the ethical committee approved on our follow-up study (NH019 2015-10-S58330), where we procure lungs of DBD and DCD donors that are refused for lung transplantation, and transport these lungs to our laboratory for research purposes. Donor data will be registered as indicated above. In this follow-up study, we will evaluate the rejected human donor lungs during EVLP which will provide insights in the mechanisms why these lungs do not meet the acceptance criteria and which lungs could be salvaged by EVLP in a future clinical EVLP program for ECD lungs. Also, we will investigate the effect of EVLP on these rejected donor lungs by CT-scanning the lung grafts prior and after EVLP. Preliminary data (presented as an abstract at the ECTTA meeting in Barcelona, Oct 2016) are depicted in Figure IV.3.

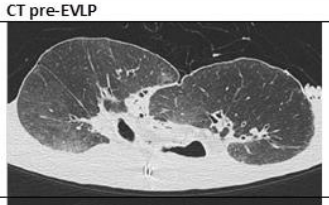
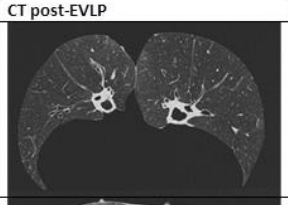
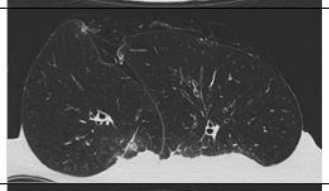
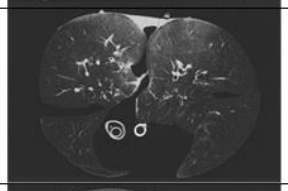
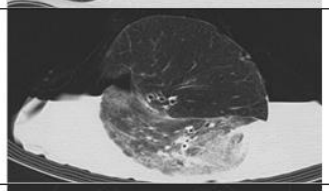
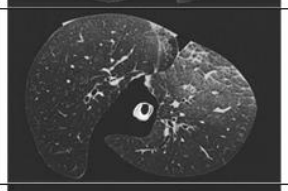
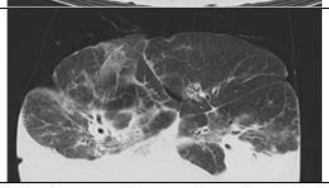
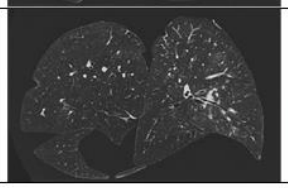
Nr°	Reason Decline	CT pre-EVLP	CT post-EVLP	EVLP
RHL1 36y Male DBD (Intra-Cerebral Bleeding)	Increased pulmonary pressures + Edema lower lobes			**EVLP Time: 4 hrs** **PaO_2/FiO_2: 610 → 614** **PVR: 279 → 302 (dynes*sec*cm^{-5})** **Ppeak: 20 → 18 (cmH_2O)**
RHL2 70y Male DCD (Trauma Capitis)	Logistical reason (no OR available)			**EVLP Time: 2 hrs** **PaO_2/FiO_2: 600 → 657** **PVR: 222 → 248 (dynes*sec*cm^{-5})** **Ppeak: 9 → 17 (cmH_2O)**
RHL3 78 Female DBD (Intra-Cerebral Bleeding)	PaO_2 210 mmHg (100% FiO_2) Atelectasis + Infiltrates lower lobes			**EVLP Time: 2.5 hrs** **PaO_2/FiO_2: 595 → 648** **PVR: 308 → 279 (dynes*sec*cm^{-5})** **Ppeak: 12 → 12 (cmH_2O)**
RHL4 33y Male DBD (Sub-arachnoidal bleeding)	PaO_2 113 mmHg (100% FiO_2) Parenchymal infiltrates			**EVLP Time: 2 hrs** **PaO_2/FiO_2: 586 → 505** **PVR: 404 → 348 (dynes*sec*cm^{-5})** **Ppeak: 10 → 10 (cmH_2O)**
DBD = donation after brain death; DCD = donation after circulatory death *EVLP parameters are depicted as: First value after rewarming (1hr) → Final value at the end of EVLP*				

Figure IV.3 – RHL1 was declined due to increased pulmonary pressures and edematous lower lobes detected during in situ donor evaluation. During EVLP, we did not detect any sign of increased pulmonary pressures (Ppeak) or vascular resistance (PVR) and CT shows a reduction in edema after EVLP. RHL2 was declined for logistical reasons since there was no OR available at the time (otherwise qualitatively good donor lung). Lungs could potentially be preserved on EVLP until an OR became available. RHL3 was declined due to bad arterial blood gases with consolidation and atelectasis in the left lower lobe. EVLP resulted in recruitment of the lungs and a decrease in infiltrates on CT scan. RHL4 was rejected based on bad arterial blood gases with parenchymal infiltrates shown on CT scan. EVLP showed a stable physiological evaluation and CT post EVLP showed a reduction in parenchymal infiltration.

Although inclusion of EVLP in several clinical program has led to an increase in the donor pool and transplant activity, the use of these donor lungs without EVLP has also been implemented with good short-term outcome compared to SCD lung transplantation. Therefore, the question remains what the real impact of EVLP could be, if lung donors declined by experienced ECD lung transplant centers are selected for EVLP recovery. With this first retrospective data analysis of unused lung donors, we identified that there is a large potential for EVLP to increase the donor pool. Preclinical studies will have to validate this hypothesis and examine the safety of accepting these lungs for transplantation.

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CHAPTER IV
EVLP POTENTIAL AND FEASIBILITY

IV.B CLINICAL IMPLEMENTATION OF EX-VIVO LUNG PERFUSION IN PEDIATRIC COMBINED LIVER-LUNG TRANSPLANTATION: A CASE REPORT

Submitted for publication:
Martens A, Pirenne J, Van Raemdonck D, Vanaudenaerde B, Vos R, Verleden GM, Verleden SE, Neyrinck AP. Sequence of combined liver-lung transplantation with normothermic lung preservation: how anesthesiologists should be involved, a case report

A) PREFACE

In this chapter, we show that EVLP not only holds great potential to increase the number and quality of transplantable donor lungs, but that it is also feasible for implementation in current clinical practice. Even in the most complex cases, such as a pediatric combined liver lung transplantation. In this case report, we preserved the lungs normothermically on a portable EVLP device, while the liver was transplanted first. Furthermore, we focus on the peri-operative period of this pediatric cystic fibrosis patient, to stimulate the community to involve anesthesiologists in the optimization of preservation strategies, since they are often confronted first with the impact of organ failure in the peri-operative period of solid organ transplantation.

B) INTRODUCTION

Combined liver-lung transplantation is considered a life-saving treatment option for well-selected cystic fibrosis patients suffering from end-stage respiratory disease with liver failure. The major cause of morbidity and mortality in cystic fibrosis (CF) patients is respiratory disease, however, liver failure associated with CF is the fourth most prevalent cause of death among these patients (1). Experience with combined liver-lung transplantation is limited and to the best of the author's knowledge, normothermic EVLP for a pediatric combined liver-lung transplantation has not been reported. Consequently, the authors show that normothermic EVLP is feasible for pediatric transplantation to limit prolonged cold ischemic time that is linked with primary graft dysfunction (PGD). However, also ischemia-reperfusion injury (IRI) of the liver can lead to PGD of the lung. IRI is inherent to the process of solid organ transplantation and has remote impact on all organ systems which may lead to serious consequences. The anesthesiologist is often confronted with the impact of IRI on the systemic physiology and is responsible of managing organ failure in the peri-operative phase. Therefore, the authors suggest to involve the anesthesiologists in the optimization of preservation strategies, including normothermic organ machine perfusion.

C) CASE REPORT

A 16-year old Caucasian female (ASA4, A Rh+, CMV-, 49.5 kg, 155 cm) was listed for a combined liver-lung transplant (written consent was obtained to publish the case report). She suffered from cystic fibrosis (genotype DF 508/Y1092X) complicated with liver cirrhosis and portal hypertension (MELD 14, CHILD PUGH B8). The portal hypertension resulted in severe hypersplenism, esophageal varices, thrombocytopenia, hypoalbuminemia and hyperammonemia for which a transjugular intrahepatic portosystemic shunt (TIPS) was applied at the age of 8 years old. Her pre-transplant forced expiratory volume in 1 second (FEV_1) and forced vital capacity (FVC) were 0.89 L (35% predicted value) and 1.98 L (67% predicted value), respectively. Prior to surgery, she had normal serum electrolytes and kidney function but suffered from an increased bleeding tendency (INR 1.7 and thrombocytopenia 45.000). The patient was hospitalized 23 days prior to the liver-lung transplant procedure for intravenous antibiotic treatment, because of persistent fever with known colonization of P. aeruginosa and A. Xylosoxidans.

After 110 days on the waiting list, a 47-year old Caucasian female brain-dead donor (hemorrhagic stroke, A Rh+, CMV-, 55kg, 165cm) was matched for liver and lung donation. The double-lung block was procured with a cold antegrade flush (4L of Organ Care System (OCS) solution plus 50mg nitroglycerin) and normothermically preserved on the OCS Lung™ device (Transmedics, Andover, MA, USA). The liver was flushed with cold IGL-1 Solution (5L) and stored on ice for 438 minutes. The rationale to transplant the liver first derives from the theoretical advantage of limiting the cold ischemic time of the liver to prevent biliary strictures, restore coagulation disorders prior to the lung transplant procedure to prevent massive transfusion, and to let the native lungs capture the liver IRI instead of the newly transplanted lungs (2).

Normothermic lung preservation

After in situ cold flush, lungs were slowly rewarmed on the Organ Care System (OCS) device in the donor hospital and kept in preservation mode for 667 minutes according to the OCS protocol. Pulmonary vascular resistance and peak airway pressure were stable throughout the entire ex vivo perfusion time with acceptable PaO_2/FiO_2 ratios (partial oxygen pressure over fractional inspired oxygen concentration, normal value >300) at the initial and final evaluation (FIGURE IV.4A). After OCS preservation, the lungs were again antegradely cold-flushed with 2 L of buffered OCS solution and split into a left and right graft for immediate implantation. The posterior side of the left lung appeared somewhat edematous (FIGURE IV.4B).

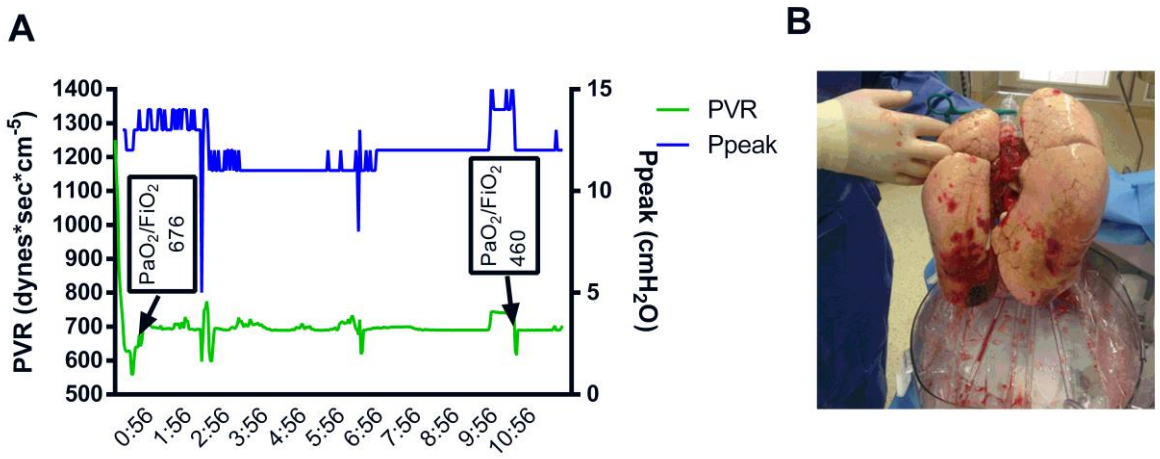

FIGURE IV.4 – A) Stable pulmonary vascular resistance (PVR) and peak airway pressure (Ppeak) during the 667 minutes of normothermic machine preservation. Initial PaO_2/FiO_2 and final PaO_2/FiO_2 ratio are shown at respectively 69 and 632 minutes. B) Dorsal aspect of the lungs after 667 minutes of normothermic machine perfusion. The left inferior lobe looks more edematous than the right lung.

Peri-operative care

Anesthesia was induced with 10µg of sufentanil and 100mg of propofol via a peripheral 18 Ga intravenous access (IV). A single lumen tube (7.0) was placed after neuromuscular blockade with rocuronium 50mg. Anesthesia was maintained with sevoflurane (2.5% inspiratory fraction) and intermittent boli of sufentanil (1µg/kg/h). Neuromuscular blockade was

maintained with rocuronium (0.5-1mg/kg). Ventilatory settings (Tidal Volume, Respiratory Rate, Positive End-Expiratory Pressure, maximum Peak Pressure, Fraction of inspired Oxygen) were adjusted according to arterial blood gases and surgical needs (single-lung ventilation). A 20Ga arterial catheter was placed in both left and right arterial catheter. And a triple lumen central venous catheter and pulmonary artery catheter (CCOmbo 744HF75, Edwards Life Sciences®) were placed in the right internal jugular vein. Finally, a gastric tube, bladder catheter, temperature probe and transesophageal ultrasound probe were inserted. Hypothermia was prevented using a Hotline (Smiths Medical), Ranger (3M) and Bair Hugger (3M) for patient warming. The patient was already under quadruple intravenous antibiotic therapy (minocycline, co-trimoxazole, tazobactam, tobramycin). The peroperative hemodynamic parameters (blood pressure, heart rate, pulmonary artery pressure, cardiac output) together with vasopressive/inotropic drugs and administered fluids are depicted in FIGURE IV.5.

First, orthotopic liver transplantation was performed via a bisubcostal incision under portocaval bypass for hemodynamic stabilization. Therefore, an extracorporeal heparin coated centrifugal pump system diverts blood from the femoral vein (20Fr) and portal vein (28Fr) to the axillary vein (15Fr). After 101’ minutes the patient was successfully weaned of portocaval bypass. Hemodynamic support with noradrenaline was increased up to 0.2 µg/kg/min during the anhepatic phase with increasing lactate values (max. 4.2mg/dl). During the reperfusion phase of the graft, lactate levels returned to their preoperative values.

After hemostasis, the incision was closed before a bilateral thoracotomy in the fifth intercostal space was performed to proceed with the sequential single lung transplant procedure. The single lumen endotracheal tube was replaced by a left-sided double lumen endotracheal tube 35 Fr. The right lung was implanted first based on the perfusion scan (42% of perfusion to right lung). After reperfusion of the first lung, pulmonary artery pressures (PAP) steadily increased (PAP_{max} 66 mmHg) and 20 ppm inhaled nitric oxide was started. During implantation of the second graft

on the left side, IRI of the first lung led to high ventilation pressures and increasing demands of hemodynamic support. There was no extra-corporeal membrane oxygenation (ECMO) applied since CO was maintained and gas exchange was acceptable, although with high doses of noradrenaline and adrenaline. This resulted in a rise in lactate levels towards the end of the procedure to a maximum of 6.2mg/dl.

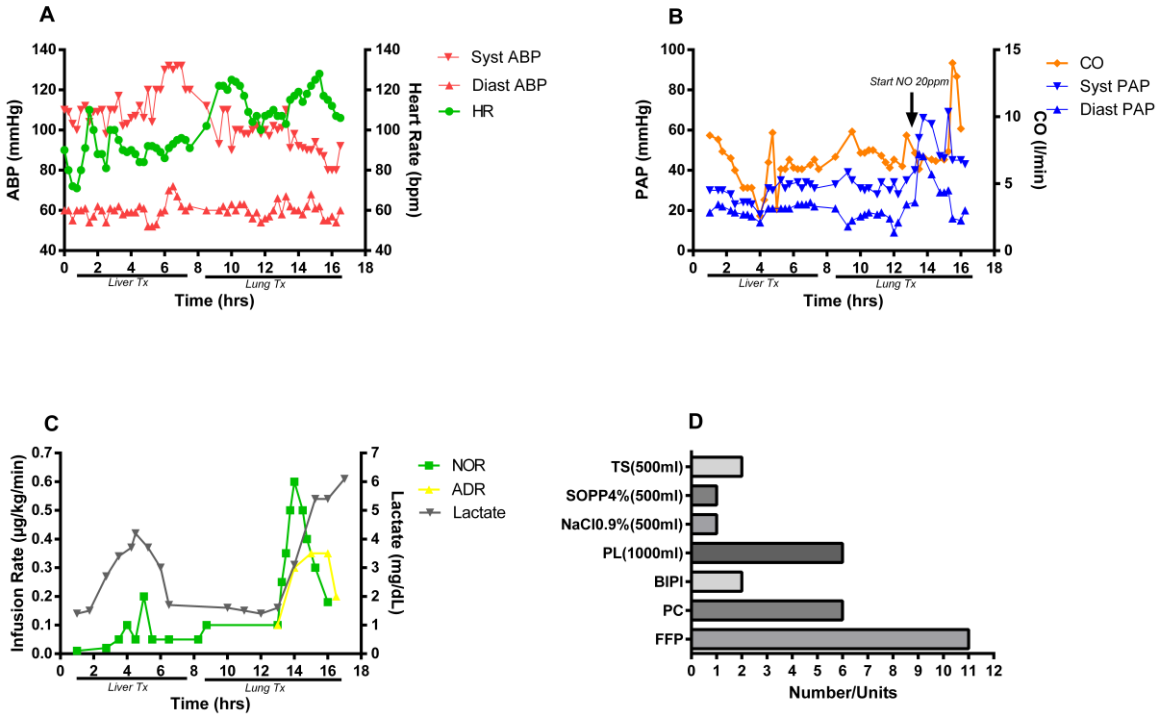

Figure IV.5 – Perioperative parameters. A) Stable arterial blood pressure (ABP) and heart rate (HR) B) pulmonary artery pressure (PAP) with preserved cardiac output (CO) under only a minimal support of noradrenaline during liver transplantation. C) Higher need for noradrenaline (NOR) and adrenaline (ADR) during the lung transplant phase with rising lactate and PAP. D) Total volume of infused fluids during the procedure

Postoperative stay Intensive Care Unit

Her postoperative stay in the intensive care unit (ICU) was 36 days during which the patient was mechanically ventilated for 8 days. Cardiovascular function was stable with minimal vasoactive and inotropic support (FIGURE IV.6A).

LIVER - The liver function disorders rapidly declined after the liver transplant with normalization of the liver function tests (FIGURE IV.6B). Duplex of the transplanted liver initially showed higher hepatic artery resistive indices due to fluid overload and IRI, which decreased upon normalisation of the fluid status. Systolic acceleration times were normal at all times.

LUNG - Reperfusion edema in the early postoperative hours after ICU admission was treated successfully with high PEEP, protective ventilation and inhaled NO. At day 5, a first spontaneous breathing trial led to extubation but because of vastly increasing work of breathing and increasing oxygen needs the patient was re-intubated after 3 hours. A second attempt on day 8 was successful. Postoperative oxygenation capacity and ventilatory support are depicted in FIGURE IV.6C. On day 24 a new air leak on the chest drain was observed and bronchoscopy confirmed limited dehiscence of the right main bronchial suture line (MDS-classification M2bD0bS1f (3)). Treatment was conservative and 6 days later, bronchoscopy could not detect a bronchopleural fistula anymore and the air leak was no longer present.

KIDNEY - Acute kidney failure stage 3 (Kidney Disease Improving Global Outcomes (KDIGO)) developed and continuous veno-venous hemofiltration (CVVH) was initiated at postoperative day 2 (FIGURE IV.6D). After 23 days, CVVH was switched to intermittent hemodialysis (IHD). After 1 month stay at the ICU, her diuresis was slightly improving to 200ml/day with increasing creatinuria as a sign of her kidney recovery and she eventually became dialysis independent, with normalization of the estimated glomerular filtration rate.

Immunosuppression was started with tracrolimus (Prograft® 2x2mg), mycophenolate mofetil (CellCept® 1.5g 2x/d) and steroids (Solumedrol® 20mg/d). However, because of an epileptic insult due to posterior reversible encephalopathy syndrome, tacrolimus was switched to cyclosporine (Neoral® 125mg 2x/d) and low-dose valproate (Depakine®) was added with full neurological recovery.

The patient is now alive and has resumed school, with normal hepatic (total bilirubin 0.74 mg/dl), pulmonary (FEV1 77%) and renal function (creatinine 0.75 mg/dl), 14 months after transplantation.

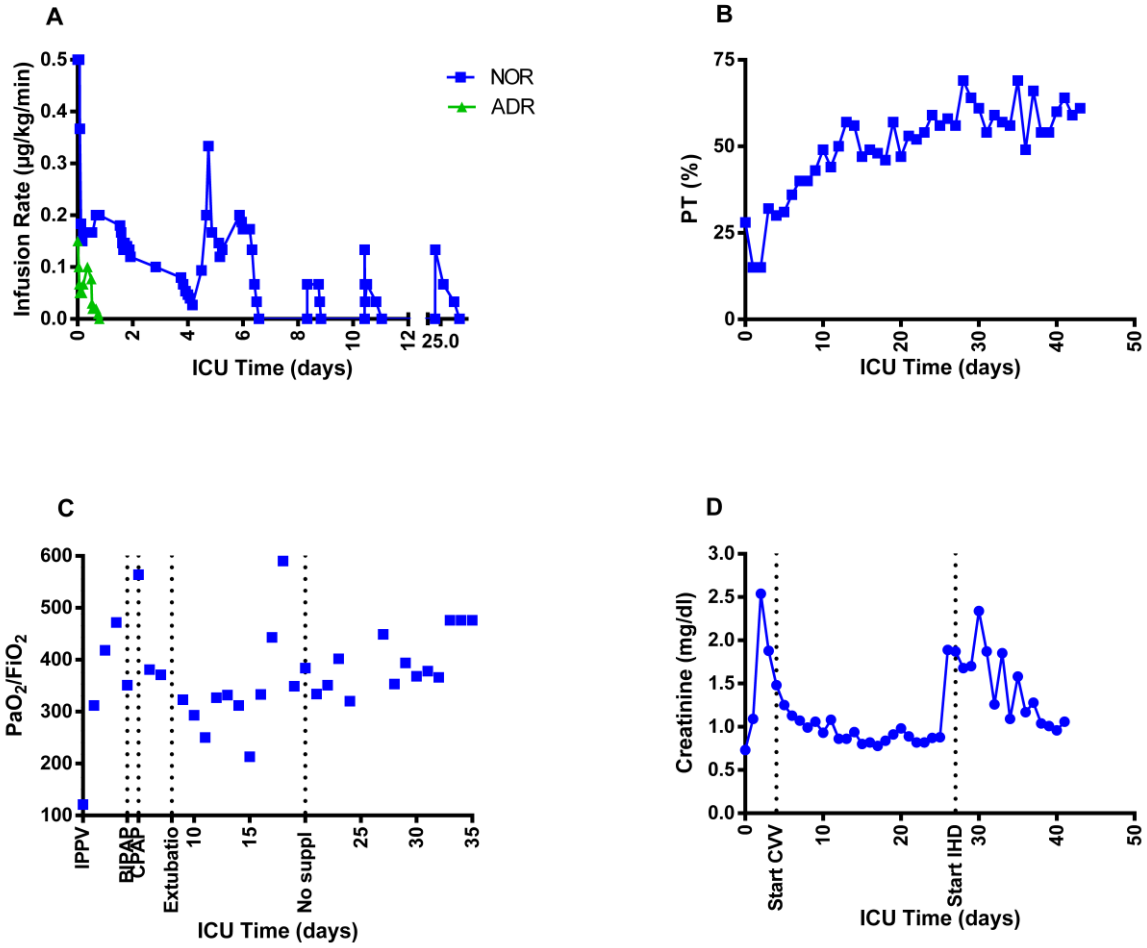

Figure IV.6 – Day 0 is the arrival at ICU. A) Hemodynamic support with adrenaline (ADR) and noradrenaline (NOR). B) Liver function resembled by Prothrombin Time (PT) gradually normalized in the postoperative period. C) Lung function is depicted as oxygenation (PaO_2/FiO_2 ratio). The patient was weaned from intermittent positive pressure ventilation (IPPV) to biphasic positive airway pressure (BIPAP) and continuous positive airway pressure ventilation (CPAP). At day 8 she was extubated and supplemental O_2 was administered with a nasal cannula until day 20. D) Acute kidney failure (peak creatinine levels) required CVVH (started at day 2), which was switched to IHD at day 22.

D) DISCUSSION

We report the first pediatric combined liver-lung transplantation with normothermic preservation of the lungs, performed in our center. Combined liver-lung transplantation is a complex procedure and is applied only in well-selected patients. We have recently reported our overall single-center experience of combined liver-lung transplantation (2). Historically, the sequence is most frequently performed as lung first, followed by liver transplantation. The main reason is the ischemic tolerable cold ischemic interval, which is believed to be shorter for lungs than livers. Occasionally, the liver has been implanted first in case of severe liver failure, applying the principle of "sickest organ first". In addition, there is also a theoretical immunological advantage to transplant the liver first (2).

The potential limitations due to the ischemic tolerance of pulmonary grafts is currently challenged, since we have better strategies to optimize cold ischemic tolerance (4) and normothermic preservation with ex-vivo lung perfusion (EVLP) has emerged as a promising technique to prolong preservation time (5). This technology is also being investigated to preserve liver grafts in normothermic conditions.

Historically, the focus and interest of anesthesia practice and research in organ preservation is limited. However, the anesthesiologist is often confronted with the impact of organ failure resulting from ischemia-reperfusion injury. This injury has a serious impact on the specific organ system and may lead to multiple systemic consequences.

In case of liver transplantation, metabolic acidosis, hyperkalemia, hypoglycemia and coagulopathy might be life-threatening complications (6). A specific issue in case of transplanting lungs after liver, is the added risk to develop additional lung injury (7). This might be related to transfusion, severe systemic inflammation, hemodynamic instability and IRI of the liver with its remote effect on the freshly transplanted lungs. A lung protective ventilation strategy is therefore crucial (8). Portopulmonary hypertension and hepatopulmonary syndrome

(9) should also be considered as underlying respiratory disorders complicating the anesthetic management.

The reperfusion injury following lung transplantation is referred to as primary graft dysfunction (PGD). PGD compromises the systemic oxygen delivery and forms a serious threat itself for other organ systems. It occurs within 72 hours after implantation and is characterized by severe high permeability pulmonary edema. Therapy for established PGD remains generally supportive and includes protective ventilation strategies, extracorporeal membrane oxygenation and selective pulmonary vasodilators such as inhaled nitric oxide (10). Despite acceptable management, we would consider earlier use of ECMO during this procedure in order to stabilize systemic oxygen delivery and hemodynamics. Peri-operative risk factors for PGD include high pulmonary artery pressures, the use of cardiopulmonary bypass, blood transfusion and increased intraoperative fluid administration (11,12).

Kidney dysfunction might compromise the outcome following both lung or liver transplantation (13,14). In case of combined organ transplantation, prolonged hemodynamic instability and secretion of toxic mediators might severely increase the risk to develop acute kidney failure.

Besides the severe challenges to manage these patients peri-operatively, we should increase our awareness and interest in these new normothermic preservation strategies. The principle for EVLP is based on an in-depth physiological evaluation outside the body, prior to implantation. Quality of the graft is assessed using perfusion parameters, ventilation parameters and oxygenation capacity (15). However, there is currently limited data available how to use the ex vivo data as a predictor for in vivo performance of the graft (16).

E) CONCLUSION

Combined liver-lung transplantation is considered a life-saving treatment option for cystic fibrosis patients suffering from end-stage respiratory disease with liver failure. However, experience with pediatric combined liver-lung transplantation is limited. Ischemia-reperfusion injury (IRI) is inherent to the process of solid organ transplantation and this injury has serious impact on all organ systems and may lead to serious consequences. The anesthesiologist is often confronted with the impact of IRI on the systemic physiology. Besides the severe challenges to manage these patients peri-operatively, we should also increase our awareness and interest in the optimization of preservation strategies, including normothermic ex-vivo lung perfusion.

We report our experience and anesthetic consequences on a case of combined liver-lung transplant with prolonged normothermic preservation of lungs on a portable ex vivo device prior to transplantation.

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CHAPTER V
EX-VIVO RECONDITIONING WITH NOBLE GASES

V.A EX-VIVO POSTCONDITIONING WITH NOBLE GASES TO ATTENUATE PULMONARY ISCHEMIA-REPERFUSION INJURY

Adapted from:

Martens A, Montoli M, Faggi G, Katz I, Pype J, Vanaudenaerde BM, et al. Argon and xenon ventilation during prolonged ex vivo lung perfusion. J Surg Res. 2016 Mar;201(1):44–52 (DOI: 10.1016/j.jss.2015.10.007)

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A) PREFACE

Ex-vivo lung perfusion (EVLP) has been put forward as the ideal platform for active improvement (reconditioning) of donor lung quality. Reconditioning could be pursued by inhalational therapy, which is an ideal route of drug delivery since the lungs are approached at the epithelial side of the alveolar membrane to tackle IRI. Noble gases have previously been shown to be organoprotective due to their anti-apoptotic and anti-inflammatory properties in other organ systems, but not in the lung. Therefore, they are of particular interest to modulate ischemia-reperfusion injury in lung transplantation. Ventilation with noble gases during EVLP allows for an optimal delivery route and immediate assessment of the impact of the reconditioning strategy prior to transplantation.

In this chapter, we investigated if noble gases (argon and xenon) could improve warm-ischemic injured donor lungs when administered by ventilation during EVLP.

B) ABSTRACT

Introduction - Evidence supports the use of ex-vivo lung perfusion (EVLP) as a platform for active reconditioning prior to lung transplantation to increase the potential donor pool and to reduce the incidence of primary graft dysfunction (PGD). A promising reconditioning strategy is the administration of inhaled noble gases based on their organoprotective effects. Our aim was to validate a porcine warm ischemic lung injury model and to investigate postconditioning with argon (Ar) or xenon (Xe) during prolonged EVLP.

Methods - Domestic pigs were divided in 4 groups (n=5/group). In the negative control (NC) group lungs were flushed immediately. In the positive control (PC) and treatment (Ar, Xe) groups, lungs were flushed after a warm ischemic interval of 2 h in situ. All grafts were evaluated and treated during normothermic EVLP for 6 h. In the control groups, lungs were ventilated with 70% N_2/30% O_2 and in the treatment groups with 70% Ar/30% O_2 or 70% Xe/30% O_2, respectively. Outcome parameters were physiological variables (PVR, Ppeak, PaO_2/FiO_2), histology, wet-to-dry weight ratio (W/D), bronchoalveolar lavage (BAL) and CT-scan.

Results - A significant difference between NC and PC for PVR, Ppeak, PaO_2/FiO_2, W/D, histology and CT-imaging was observed. No significant differences between the injury group (PC) and the treatment groups (Ar, Xe) were found.

Conclusion - We validated a reproducible prolonged 6 h EVLP model with 2 h of warm ischemia and described the physiological changes over time. In this model, ventilation during EVLP with Ar or Xe administered post-injury, did not improve graft function.

C) INTRODUCTION

Lung transplant programs are being hampered by organ shortage due to a low recovery rate of donor lungs from multi-organ donors (1). Successful efforts have been made to increase the donor pool including the use of donors after circulatory death (DCD) and extended-criteria donors (ECD) (2,3). However, further expansion with acceptable grafts is still requisite. In addition, the problem of primary graft dysfunction (PGD), resulting from ischemia-reperfusion injury (IRI), decreases early post-transplant outcome (4,5). To enlarge the donor pool and to improve outcome, active resuscitation of donor organs prior to transplantation is a promising strategy.

Normothermic ex-vivo lung perfusion (EVLP) is entering the clinical reality as a tool for graft evaluation and preservation (6). This technique was developed to evaluate DCD donor lungs prior to transplantation (7). EVLP was further successfully applied to assess and recruit previously rejected organs (8). Currently, research is investigating the potential to actively recondition lung grafts with EVLP (9), serving as a platform to stimulate repair mechanisms while organs are metabolically active. Prolonged and stable perfusion times are essential conditions for EVLP rehabilitation, which can be extended up to 12 hours (10).

Inhalational therapy for lung rehabilitation is an excellent administration route, and research on inhalational therapy like carbon monoxide (11) and hydrogen therapy (12) have already been investigated. However, the effects of noble gases on IRI have not been investigated so far. Noble gases, including argon (Ar) and xenon (Xe), are chemically inert, but exhibit biological effects (13,14). Various in vitro and in vivo injury models in the brain (15,16), myocardium (17) and kidneys (18,19) have shown a protective effect of argon and xenon attributed to anti-apoptotic and anti-inflammatory properties (20–25). The problem with Xe is its scarcity (0.9 ppm), while in contrast Ar is the third most abundant gas in the earth's atmosphere (9300 ppm). Therefore, the use of Ar treatment could be of higher economic benefit.

Potential organ-protective effects on pulmonary grafts have not been explored and IRI might be a perfect target for noble gas treatment. A major advantage is the possible administration of these gases through ventilation. In addition, higher concentrations in the gas phase can be administered during EVLP without additional risk for the recipient.

This study aims to investigate the potential of pulmonary allograft reconditioning using ex-vivo noble gas treatment during 6 hours of normothermic EVLP in a 2-hour warm ischemic injury model.

D) METHODS

Animals

Domestic male pigs (Topigs 20, 36-42 kg) were used. Local ethical approval was obtained at the research institute (NTS P043/2014). All animals received humane care in compliance with the 'Principles of Laboratory Animal Care' formulated by the National Society for Medical Research and the 'Guide for the Care and Use of Laboratory Animals' prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (NIH Publication No. 86-23, revised 1996).

Animal anesthesia and baseline

Anesthesia was induced by an intramuscular injection with 5 mg/kg Zoletil 100® (Virbac, Carros, France) and 3 mg/kg Xyl-M 2%® (V.M.D., Arendonk, Belgium). Muscle relaxation and analgesia were maintained with 2 mg pancuronium and 20 µg/kg/h of fentanyl. Continuous intravenous infusion of 10 mg/kg/h propofol was used for anesthesia maintenance. Animals were intubated with a 7.0 mm endotracheal tube and ventilated (Aestiva 3000, GE Healthcare Europe GmbH, Little Chalfont, United Kingdom) with a tidal volume (TV) of 8 ml/kg, PEEP of 5 cmH_2O and FiO_2 of 30%. Respiratory rate was adjusted to $ETCO_2$ (45-55 mmHg). Invasive blood pressure was monitored. Animals received 1 g cefazolin, 500 mg solumedrol and 300 IU/kg of heparin.

Study groups

Animals were divided in 4 groups (n=5/group): negative control (NC); positive control (PC); argon group (Ar) and xenon group (Xe).

In the PC and treatment groups, circulatory and respiratory arrest was induced by myocardial fibrillation with an electrical pulse generator and ventilator disconnection. Animals were left

untouched at room temperature (warm ischemic interval of 120 min in situ). In the NC group, no warm ischemia was induced and lungs were procured immediately.

Procurement of donor lungs

A median sternotomy was performed following baseline assessment in NC, and 15 min prior to completion of warm ischemia in PC, Ar, and Xe. The pulmonary artery and caval veins were encircled and a purse-string was sutured on the right ventricular outflow tract to secure the 20 Fr flush cannula in the pulmonary trunk. Following inflow occlusion, lungs were cold flushed (4 °C) in an antegrade way with 50 mL/kg THAM-buffered Perfadex® (XVIVO Perfusion AB, Goteborg, Sweden). To optimize flush conditions, lungs were protectively ventilated (inspiratory pressure of 15 cmH_2O and PEEP of 5 cmH_2O). After explantation, an additional retrograde flush (1 L) was performed and the lungs were cannulated with the XVIVO Lung Cannula Set™ with closed atrium. An endotracheal tube of 8.0 mm was secured in the trachea.

Perfusate

The perfusate (1.5 L) was composed of THAM-buffered Perfadex® with 70 g/l of human albumin (C.A.F.-D.C.F., Neder-Over-Heembeek, Belgium). Also, 2.5 g glucose, 1 g cefazolin, 500 mg solumedrol, 50 mEq sodium bicarbonate, 0.18 g calcium and 30 IU of insulin were added. Baseline samples of the priming solution were analyzed.

Ex-vivo lung perfusion

Our EVLP circuit consisted of a centrifugal pump, a gas exchanger, a reservoir and a leukocyte filter as previously described (26). Oxygenated normothermic flow to both lungs (37°C) was gradually increased over one hour to a target of 40% of the estimated cardiac output (0.1 mL/kg of the body weight). Left atrial pressure was kept between 3 and 5 mmHg (27). Once the effluent reached 34°C, volume-controlled ventilation was started with a TV of 7 mL/kg, 7 breaths/min and PEEP 5 cmH_2O. Grafts were ventilated with 30% oxygen in each group and recruited hourly. According to the study group, 70% nitrogen (N_2, PC), Ar or Xe were added (Air Liquide

Santé France, Gentilly, France). The gas exchanger in the circuit was switched to a mixture of oxygen, CO_2 and nitrogen to compose a mixed venous gas concentration at the inflow. Recording of pulmonary vascular resistance (PVR[dynes/sec/cm^{-5}]=((PAP[mmHg]-LAP[mmHg])*80)/Flow[L/min], Peak airway pressures (Ppeak) and blood gases for oxygenation were performed hourly. Total EVLP time was 6 hours; however, experiments were prematurely ended when the reservoir was empty due to lung edema formation. EVLP was aborted when the reservoir went empty instead of refilling it with solution to avoid dilution of toxins and inflammatory mediators which could alter the outcome.

Tissue sampling

At end of EVLP, following biopsies were taken: right upper lobe (RUL); lower part of the right lower lobe (RLL/L); middle part of the right lower lobe (RLL/M). Biopsies were fixed in 6% formaldehyde, embedded in paraffin and stained with hematoxylin-eosin. The presence of alveolar necrosis, alveolar macrophages, alveolar neutrophils and bronchial inflammation was scored by a blinded reviewer (28). The mean severity score of the 3 samples per lung was analyzed. Wet-to-dry weight ratio (W/D) of a second RLL sample was determined to quantify lung edema (29).

A 30 cc saline bronchoalveolar lavage (BAL) was performed in duplicate in the right middle lobe as previously described (28). Returned fractions were pooled and a cytospin was stained with Diff-Quick (Dade Behring, Newark, NJ) to perform differential cell counts.

Finally, the left lung was inflated at 25 cmH_2O with an F_iO_2 of 30% and frozen in liquid nitrogen vapor to allow computed tomography (CT)-scanning and radiographic analysis as described previously(30,31). This imaging analysis yielded measures of ground glass opacity as a general marker of acute alveolar injury and septal thickening and consolidation as markers of lung edema. These variables were scored by a blinded reviewer.

Statistical Analysis

Data analysis was performed with the statistical software package Graphpad Prism 4 (GraphPad Software Inc., CA, USA). All data are expressed as median ± interquartile range (IQR). In the cases where lungs could not sustain the full 6 hours of EVLP, data points recorded in the next hours after the premature end of EVLP were considered the same as the last data point available to allow comparison at all evaluation points. Data were compared with a non-parametric 1-way ANOVA (Kruskal-Wallis) for one time-point and a repeated measures 2-way ANOVA for evaluation of the variables in time. When the overall p-value was significant, a Dunn's multiple comparison test (1-way ANOVA) or Bonferroni post-hoc test (2-way ANOVA) was performed, comparing all groups to the PC group. The level of significance was set at $p < 0.05$.

E) RESULTS

Study Groups

Baseline parameters of donor animals and composition of the priming solution were comparable between all groups (Table V.1).

Table V.1 – Comparable baseline parameters and perfusate composition.

Variables	NC (n=5)	PC (n=5)	Ar (n=5)	Xe (n=5)	p value
Donor					
Weight, kg	40 (38 - 44)	42 (39 - 43)	42 (39 - 43)	39 (36 - 40)	0.18
TV, mL/kg	7.8 (7.7 - 8.1)	7.7 (7.6 - 8.2)	7.9 (7.7 - 8.2)	8.2 (7.8 - 8.6)	0.41
HR, bpm	113 (98 - 124)	107 (89 - 116)	104 (97 - 137)	109 (86 - 117)	0.82
MAP, mmHg	96 (89 - 104)	84 (82 - 124)	114 (103 - 125)	88 (81 - 107)	0.19
Ppeak, cmH_2O	18 (16 - 20)	18 (16 - 19)	19 (18 - 21)	20 (18 - 22)	0.37
PaO_2/FiO_2	403 (370 - 460)	403 (382 - 428)	400 (368 - 442)	403 (390 - 417)	0.96
WBC * 10^6	15 (11 - 18)	16 (12 - 20)	17 (11 - 19)	18 (11 - 19)	0.92
Hct, %	35 (35 - 37)	36 (33 - 37)	36 (33 - 39)	38 (35 - 39)	0.40
Perfusate					
Alb, g/L	63 (61 - 65)	66 (63 - 67)	65 (63 - 66)	64 (60 - 68)	0.63
Osm, mmol/kg H_2O	308 (307 - 310)	309 (308 - 309)	308 (306 - 311)	312 (307 - 314)	0.50
Na^+, mmol/L	151 (150 - 153)	152 (151 - 153)	152 (150 - 153)	151 (150 - 154)	0.86
K^+, mmol/L	3.7 (3.6 - 3.8)	3.6 (3.6 - 3.7)	3.7 (3.6 - 3.7)	3.7 (3.7 - 3.8)	0.27
Cl^-, mmol/L	106 (106 - 108)	106 (106 - 106)	106 (106 - 108)	106 (106 - 107)	0.71
HCO_3^-, mmol/L	26 (25 - 27)	27 (25 - 27)	25 (25 - 25)	26 (25 - 27)	0.17
Ca^{2+}, mmol/L	0.53 (0.49 - 0.54)	0.50 (0.49 - 0.54)	0.49 (0.48 - 0.54)	0.51 (0.49 - 0.57)	0.78
Gluc, mg/dL	175 (170 - 177)	177 (175 - 184)	178 (169 - 181)	172 (169 - 186)	0.64

Data are expressed as median (IQR); 1-way ANOVA for group comparison (Kruskal-Wallis)
TV = tidal volume; HR = heart rate; MAP = Mean Arterial Pressure; Ppeak = Peak Airway Pressure; WBC = total white blood cell count; Hct = haematocrit; Alb = albumine; Osm = osmolality; Na = sodium; K = potassium; Cl = chloride; Bic = bicarbonate; Ca = calcium; Gluc = glucose

Graft Survival

For all experiments in the NC group, EVLP could be performed for 6 hours. However, in the other groups, there was a drop-out (4 drop-outs in PC and Ar; 2 drop-outs in Xe) due to extensive lung edema (Figure V.1).

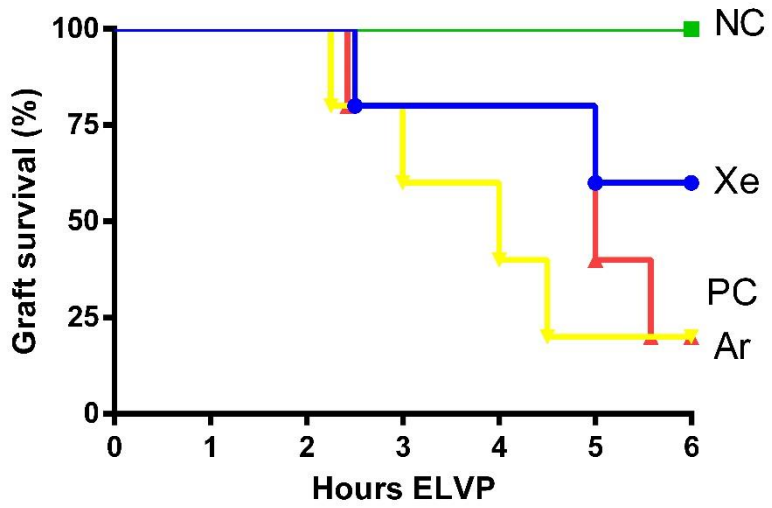

Figure V.1 – Proportional survival of lungs on EVLP. No drop out in the NC group, 2 early drop outs in Xe Group, 4 early drop outs in PC and Ar Group.

Functional assessment of pulmonary grafts during EVLP

Ppeak gradually declined in the NC group (Figure V.2). In PC and treatment groups, Ppeak initially declined but deteriorated after 3 hours. At 5 and 6 hours, Ppeak was significantly different between NC and PC. No differences in Ppeak were found between the treatment groups and PC.

PaO_2/FiO_2 ratio was stable in the NC group and clearly declined in the PC and treatment groups, however, did not reach the level of significance (Figure V.2). At the end of EVLP, oxygenation was significantly different between the NC and PC group ($p<0.05$). No effect was observed in the treatment groups.

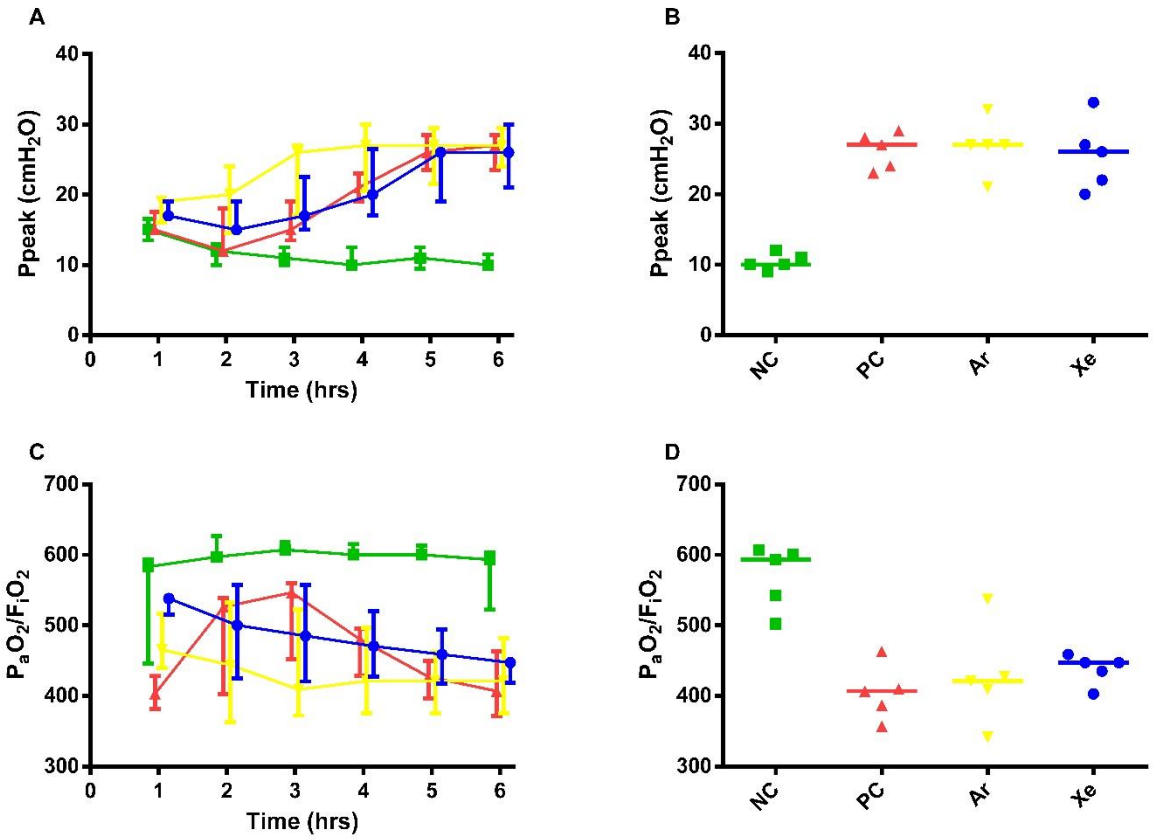

Figure V.2 – Assessment of functional parameters during EVLP. All data are depicted as median ± IQR. A, C analyzed with repeated measures 2-way ANOVA. B, D analyzed with non-parametric 1-way ANOVA. Time 0 represents start of perfusion on EVLP; time 1 is the first evaluation moment.

Stable Ppeak in NC, increased Ppeak in PC ($p<0.05$ hrs 5, 6), Ar (NS), Xe (NS)

Ppeak at the end of EVLP significantly differs between NC and PC ($p<0.05$), no difference between PC and Ar, Xe (NS)

Stable PaO2/FiO2 in NC, decreased P/F in PC (NS), Ar (Ns), Xe (NS)

PaO2/FiO2 at the end of EVLP significantly differs between NC and PC ($p<0.05$), no difference between PC and Ar, Xe (NS)

At first evaluation (1h of EVLP), PVR was similar in all groups (Figure V.3). In NC, PVR was stable throughout the whole experiment. However, in PC, Ar and Xe, PVR gradually increased, although not statistically significant. Comparing PVR at the end of EVLP, a significant difference between NC and PC ($p<0.05$) was present. No significant differences were found between the treatment groups and the PC group. Both pump flow (l/min) and measured pulmonary artery pressure (mmHg) are depicted for all groups (Figure V.3).

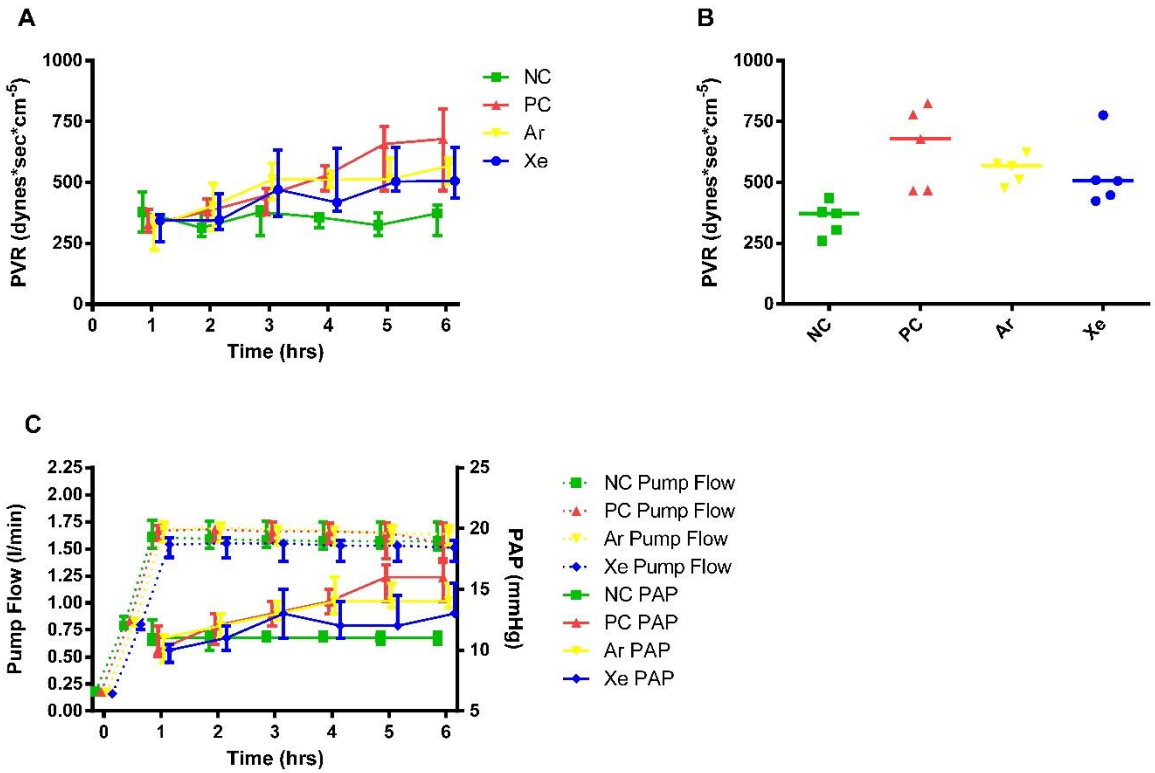

Figure V.3 – Assessment of pulmonary vascular resistance (PVR). All data are depicted as median ± IQR. A was analyzed with repeated measures 2-way ANOVA. B was analyzed with non-parametric 1-way ANOVA. Time 0 represents start of perfusion on EVLP; time 1 is the first evaluation moment.

Stable PVR in NC, increased PVR in PC (NS), Ar (NS), Xe (NS)

PVR at the end of EVLP significantly differs between NC and PC ($p<0.05$), no difference between PC and Ar, Xe (NS)

Evaluation of pump flow (40% of the estimated cardiac output) and the measured pulmonary artery pressure (PAP)

Assessment of pulmonary edema and BAL-analysis

A significant difference in W/D between NC and PC was observed, validating our injury model ($p<0.05$). However, no treatment effect of Ar or Xe was observed (Figure V.4A). No differences in BAL analysis of macrophages, neutrophils or lymphocytes were found (Figure V.4B).

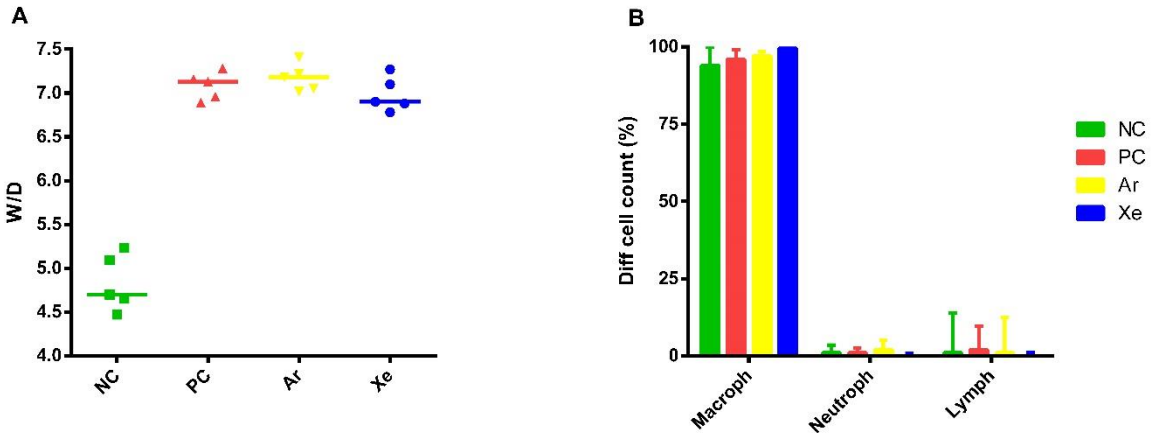

Figure V.4 – Assessment of lung edema and BAL fluid analysis. All data are depicted as median ± IQR
Non-parametric 1-way ANOVA of W/D shows significant difference between NC an PC ($p<0.05$) but no difference between PC and Ar, Xe (NS)
2-way ANOVA of BAL fluid shows no significant difference (NS)

Histology and Imaging

Histology (Figure V.5A) indicated the absence of alveolar necrosis in the NC group in contrast to all other groups ($p<0.05$). Histological sections of all groups are shown in Figure V.6. Other parameters such as the presence of alveolar macrophages, neutrophils and bronchial inflammation were not significantly different between groups. CT-imaging analysis (Figure V.5B) showed that NC and PC groups significantly differed in the presence of ground glass opacities ($p<0.01$) and septal thickening ($p<0.05$), but not in the amount of consolidation. No differences in these CT-parameters were measured between the PC and treatment groups.

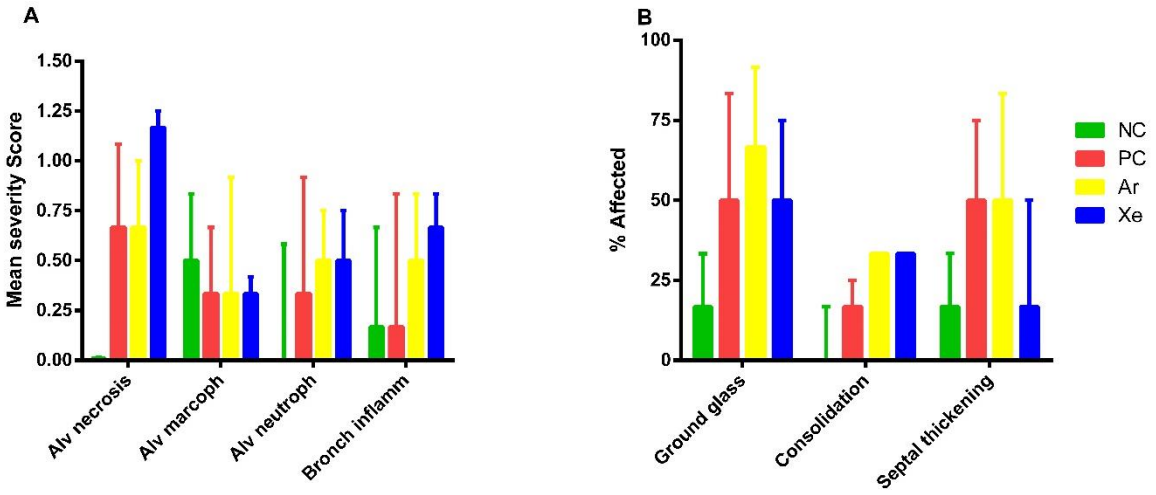

Figure V.5 – Analysis of histology-scoring and CT-imaging. All data are depicted as median ± IQR, both graphs were analyzed with a 2-way ANOVA

The mean of the histology scoring in the 3 biopsies (score range 0-3) shows significant difference between NC and PC, Ar, Xe regarding presence of alveolar necrosis ($p<0.05$). No differences were found in the other variables.

Scoring analysis of CT-imaging shows significant difference between NC and PC regarding ground glass opacities ($p<0.01$) and septal thickening ($p<0.05$), not for consolidation (NS). No significant differences were shown between PC and treatment groups (NS).

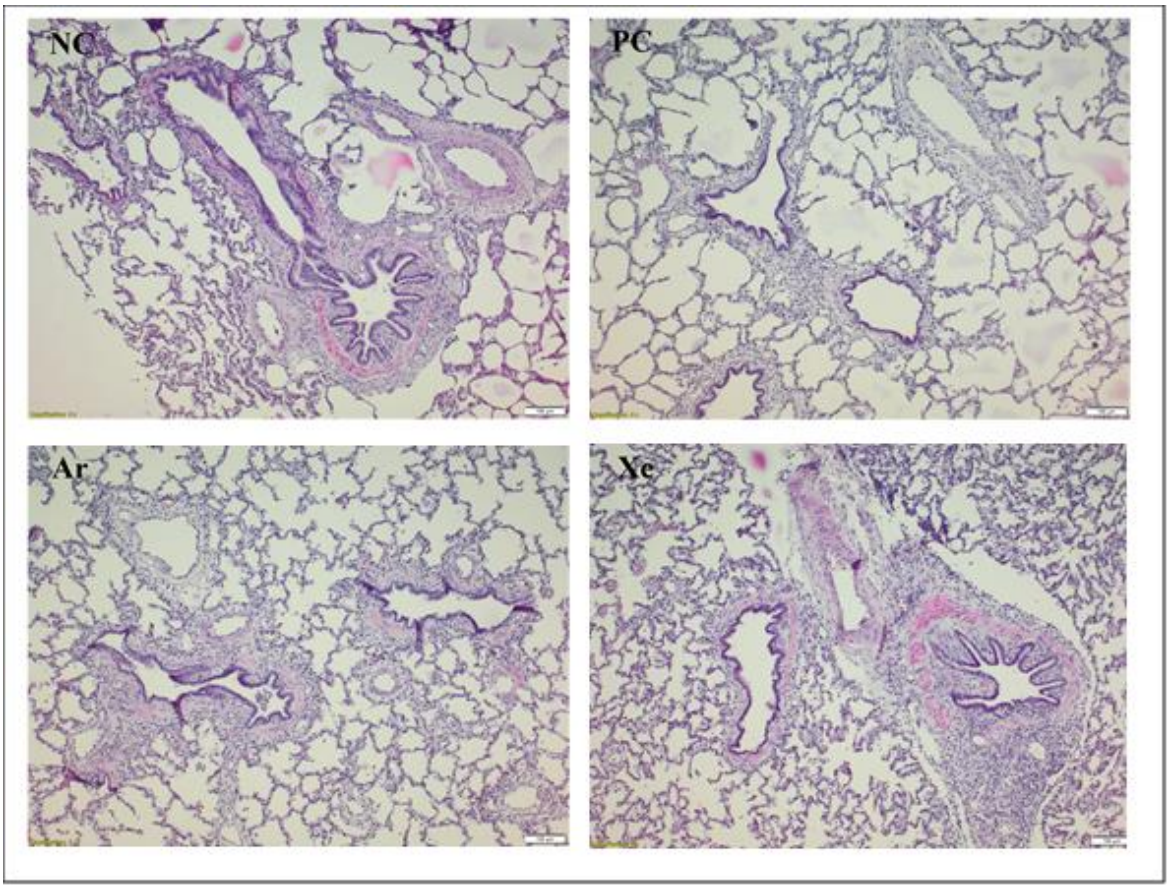

Figure V.6 – Histological sections of lung tissue in NC, PC, Ar and Xe group.

F) DISCUSSION

In this study we have demonstrated that xenon and argon ventilation at reperfusion could not significantly reduce the development of IRI during EVLP in a pig model with 2 hours of warm ischemia.

To our knowledge, we are the first to investigate the potential effect of noble gases on pulmonary IRI. More than 15 years have passed since the first beneficial effect of noble gas therapy has been reported (6). Since then, several organoprotective effects have been described. Most evidence is derived from cerebral or myocardial ischemic injury studies, both in vitro (32–35) and in vivo (36–39). On the one hand, the heterogeneity in these data makes it difficult to extrapolate them to other organ systems. On the other hand, clear evidence is provided by these models to reduce infarct size and improve neurological performance by exposure to noble gases. Also in the field of transplantation, a rat model has shown improved functional and morphological outcome in kidney transplantation after perfusion with noble gas saturated fluid (18,19). These protective effects, however, could not be demonstrated in our model.

A first reason to explain our findings is the choice of a postconditioning setting during EVLP alone. This ex-vivo postconditioning strategy has many advantages since we can apply and monitor the treatment outside the donor (following procurement) and prior to implantation in the recipient. We believe this procedure would be the most feasible for a future translation to clinical practice.

Normothermic conditions allow for different repair mechanisms to be activated. Experimental EVLP treatments include adenoviral vectors to transfer IL-10 (40) and the addition of steroids (41), adenosine-agonists (42), high doses of antibiotics (43) or surfactant (44). Clinically, the administration of fibrinolytics (45,46) has been performed, but it has limited indications. In our study, the lack of response might be due to the introduction of the noble gas only after the IRI insult to the lung. That is: after the warm ischemic interval and even after reperfusion was

started. Indeed, ventilation was only started once the outflow temperature reached 34°C and thus, reperfusion injury was already initiated. Other injury models studying organoprotective effects of noble gases have included pre-injury exposure (13,21,35,47). The rationale for postconditioning is our extensive experience with DCD lung transplantation (26,48,49) and the potential of therapies in the ex-vivo setting.

Secondly, the 2-hour warm ischemic interval is longer than restricted intervals of 60 min by other groups (50–52). We have previously demonstrated that warm ischemia is tolerable for up to 90 minutes (49) and a 2-hour interval was chosen for substantial injury based on previous validation of this model (53). The use of this extended warm ischemic interval of 120 minutes in our study design is further supported by other investigators (54,55). However, our current data indicate excessive structural damage with findings of alveolar necrosis which is potentially unsuitable as a substrate for noble gas rehabilitation. This was further supported by the observation that in some experiments excessive edema limited perfusion time. Continuous infusion of fresh perfusate to prevent the reservoir of running out of perfusion solution would be an excellent way of preventing drop-out and allow recalculation of diluted metabolites in future experiments. Based on these results, we discourage the use of a 2-hour warm ischemic interval in the use of prolonged ex-vivo lung perfusion for graft reconditioning in a future experimental study design. However, extensive reperfusion injury can also occur in clinical practice and the absence of an improvement in graft function or decrease in lung edema formation merely shows that this extensive injury cannot be reversed by noble gas ventilation.

Thirdly, the nature of reperfusion injury using acellular perfusate (Steen® solution) in comparison to blood (in vivo IRI) should be considered to explain some findings. Due to the absence of leukocytes, the inflammatory injury might be damped as seen on BAL analysis. The flow of oxygenated perfusate in the lung vasculature does mimic IRI and also induces mechanical shear stress and ROS that play a central role in the development of PGD (4,56–59).

However, the nature of the ROS introduced might be slightly different since Steen solution has some antioxidant activity (60). Furthermore, differences in viscosity and density, as well as the continuous flow with the centrifugal pump, will result in a different shear stress compared to in-vivo IRI.

Finally, the concentration of noble gas was limited to 70%. In this study, an F_iO_2 of 30% was chosen which is within the wide range of 12 to 50% advocated in other EVLP protocols (6). Future studies might modify the FiO_2 and noble gas concentration to investigate dose-response effects. Data on biological dose-responses are also limited in other organs (25).

Interpretation of the ventilator parameters is complex using gas mixtures with different physico-chemical properties. Ppeak is a common variable used to evaluate lung quality during EVLP; however, it is problematic to use when ventilation is performed using different gas mixtures because all else being equal, the gas mixture with higher density and viscosity will result in a greater Ppeak (61). Furthermore, common ventilators are not calibrated for noble gas mixtures. We measured the ventilator variables at the proximal end of the endotracheal tube using the IntelliVue 60 spirometry module (Philips Healthcare, DA Best, The Netherlands). Since this module is calibrated for 100% oxygen, volumes had to be corrected for the density of each specific gas with the following formula: $TV_{measured}=TV_{actual}*\sqrt{(\rho_{test\ gas}/\rho_{100\%O2})}$. The density for $100\%O_2$, $70\%N_2/30\%O_2$, $70\%Ar/30\%O_2$ and $70\%Xe/30\%O_2$ are 1.257, 1.148, 1.477 and 3.991 kg/m^3, respectively (62,63). By correcting the tidal volumes, the actual tidal volume delivered in the lung during EVLP was equal in all experiments: 7ml/kg. The pressure drop across the endotracheal tube is also function of the gas and thus should be omitted from the peak pressure measurement if we want to know the actual peak airway pressures. The inspiratory flow of 5.9±0.4 l/min (mean ± SD), which can be calculated based on TV, RR and I:E ratio, results in a pressure drop across the endotracheal tube of 0.41±0.03, 0.53±0.04 and 0.63±0.05 cmH_2O for $70\%N_2/30\%O_2$, $70\%Ar/30\%O_2$ and $70\%Xe/30\%O_2$, respectively. Since these pressure drops

are not significantly different for the used gas mixtures, we chose not to correct our measured ventilator pressures and displayed the measured values only. In future research different methods to assess aerodynamics should be used instead of Ppeak.

The strength of our study design is the inclusion of positive and negative control groups to enable rigid validation of the injury model, and prolonging EVLP up to 6 hours. These conditions have not been studied experimentally in combination with different injury models. Despite increasing experience with EVLP (64,65), consensus about the value of the physiological parameters is still required to predict post-transplant performance (6). We observed that the first parameter to decline was Ppeak (in contrast to PVR and P/F) after a minimum reperfusion time of 4 hours, despite extensive injury.

In conclusion, in this study we have validated a reproducible prolonged EVLP model with a 2-hour warm ischemic interval and demonstrated that Ppeak, PVR and P/F could discriminate between non-injured and injured lungs. This was validated with W/D, histology and CT-imaging. In addition, we are the first to report on noble gas therapy in lung transplantation. Treatment with Ar or Xe post-injury did not improve graft function in our model of prolonged EVLP. However, a positive effect might be missed due to the severity of the warm ischemic injury inducing extensive and probably irreversible damage.

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CHAPTER V
EX-VIVO RECONDITIONING WITH NOBLE GASES

V.B PRE-, PER-, AND POSTCONDITIONING OF LUNG GRAFTS WITH ARGON TO REDUCE ISCHEMIA-REPERFUSION INJURY

Adapted from:
Martens A, Ordies S, Vanaudenaerde B, Verleden SE, Vos R, Verleden GE, Verbeken E, Van Raemdonck D, Claes S, Schols D, Chalopin M, Katz I, Farjot G, Neyrinck AP. Maximal argon exposure of porcine lung grafts to attenuate ischemia-reperfusion injury.
Accepted for publication in Medical Gas Research

A) PREFACE

In the previous chapter, we have investigated if postconditioning with noble gases during EVLP, could reduce ischemia-reperfusion injury (IRI). However, no beneficial effect was seen on the lung physiology of warm-ischemic injured lungs when exposed to argon or xenon after the ischemic insult on EVLP (postconditioning effect). To investigate a potential interventional effect of noble gases in lung transplantation, we therefore prolonged the exposure time to the noble gas to preconditioning (prior to the ischemic injury), perconditioning (during the ischemic injury) and postconditioning (after the ischemic injury) instead of postconditioning alone. In addition, in this study we investigated the treatment effect on cold-ischemic injury rather than warm ischemia, since our previous ischemic injury model with 2 hours of warm ischemia seemed too severe to study IRI modulation. Finally, in this model we studied the effect of Ar alone given its wider application potential due to a reduced production cost compared to Xe.

B) ABSTRACT

Background - Argon (Ar) is a noble gas with known organoprotective effects in rodents and, in in vitro models. In a previous study we could not detect a postconditioning effect of Ar during ex-vivo lung perfusion (EVLP) on induced warm-ischemic injury in a porcine model. In this study, we further investigated a prolonged exposure to Ar to decrease cold ischemia-reperfusion injury after lung transplantation with EVLP assessment (porcine model).

Methods - Domestic pigs (n=6/group) were pre-conditioned for 6 hours with 21% O_2 and 79% N_2 (CONTR) or 79% Ar (ARG). Subsequently, lungs were cold flushed and stored inflated on ice for 18 hours with the same gas mixtures. Next, lungs were perfused on ex-vivo lung perfusion (EVLP) (acellular) for 4 hours while ventilated with 12% O_2 and 88% N_2 (CONTR group) or 88% Ar (ARG group). The perfusate was saturated with the same gas mixture but with the addition of CO_2 to an end-tidal CO_2 of 35-45 mmHg. The saturated perfusate was drained and lungs were perfused with whole blood for an additional 2 hours on EVLP.

Results - Evaluation at the end of EVLP did not reveal a significant effect on the physiologic parameters by a prolonged exposure to Ar. Also wet-to-dry weight ratio did not improve in the ARG group.

Conclusion - Although in other organ systems a protective effect of Ar has been shown, we did not detect a beneficial effect of a high concentration of Ar on cold pulmonary ischemia-reperfusion injury after prolonged exposure to the noble gas.

C) INTRODUCTION

Lung transplantation is still hampered by a shortage of transplantable donor grafts, and those grafts that are available are often of limited quality (1). More and more, extended-criteria donor lungs are being used for organ transplantation. And although there is a similar 1-year survival (2), there is a higher incidence of severe primary graft dysfunction (PGD) among recipients of an extended-criteria donor lung (3). PGD is the end-result of ischemia-reperfusion injury (IRI) attacking the integrity of the alveolar membrane leading to pulmonary edema and impaired oxygenation (4,5). Severe PGD is associated with an impaired short-term and long-term outcome (6). Therefore, we have to tackle the development PGD by improving donor lung quality, which is referred to as "reconditioning". Organ reconditioning can be achieved at three different stages: prior to organ injury (preconditioning), during organ injury (perconditioning) or after organ injury (postconditioning). Preconditioning is known as classical donor management, perconditioning involves optimization of the "out of body" time (preservation) and postconditioning is seen as strategies after reinstallation of perfusion.

Our previous study which investigated a postconditioning effect of argon (Ar) (and xenon (Xe)) on pulmonary ischemia-reperfusion injury did not reveal a reconditioning effect (7); although beneficial postconditioning effects of Ar have been shown in other injury models such as cardiac arrest (8,9), brain injury (10), and neonatal asphyxia (11). Noble gases Ar and Xe have also previously been investigated as a reconditioning strategy in IRI kidney transplant models with beneficial effects on graft function after exposure to these noble gases (12,13). But since the protective effect of Ar seems to be dose-dependent (14), a higher and longer exposure to Ar to protect against pulmonary ischemia reperfusion injury seemed justified. A prolonged exposure to Ar can be achieved by introducing the gas earlier in the process of organ donation (15). During prolonged ventilation of the donor, Ar might protect and precondition the pulmonary graft prior to the ischemic insult ("preconditioning"). In addition, the lung can be

inflated with the potentially protective gas mixture during cold storage ("perconditioning"). Ventilating the donor lung prior to transplantation, in normothermic conditions with inhalational therapy is feasible due to the technique of ex-vivo lung perfusion (EVLP) (16,17). Also, the perfusion solution on EVLP could be saturated with Ar, such has been used with success in experimental perfusion of kidneys preceding transplantation (12,13). Replacing N_2 with Ar in the gas mixture for the ventilator and for the gas exchanger in the EVLP system, results in a dual exposure of the pulmonary graft to Ar, at both the endothelial and epithelial side ("postconditioning").

This study aimed to investigate the effect of Ar on pulmonary ischemia-reperfusion injury when administered prior (preconditioning), during (perconditioning) and after (postconditioning) the ischemic insult to donor lungs. Normothermic EVLP was used to evaluate the treatment effect on graft function.

D) METHODS

Animals

Domestic pigs (35.8±1.6 kg) were used. Local ethical approval was obtained at the research institute (P043/2014). All animals received humane care in compliance with the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research and the "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (NIH Publication No. 86-23, revised 1996).

Animal anesthesia and study groups

Anesthesia was induced with an intramuscular injection of 5 mg/kg Zoletil 100 (Virbac, Carros, France) and 3 mg/kg Xyl-M 2% (VMD, Arendonk, Belgium). After placement of an 18GA peripheral line (v. auricularis) muscle relaxation and analgesia were maintained with 2 mg pancuronium boli and 20 µg/kg/h fentanyl. Anesthesia was maintained with continuous infusion of 7-10 mg/kg/h propofol. Animals were intubated with a 7.0 mm endotracheal tube and ventilated with a tidal volume (TV) of 8 ml/kg and 5 cmH_2O positive end expiratory pressure (PEEP). Respiratory rate was adjusted to obtain an end-tidal carbon dioxide ($ETCO_2$) of 35-45 mmHg. Invasive arterial blood pressure (ABP) was measured in the right carotid artery and a 7.5 Fr Swan Ganz catheter (Edwards Lifesciences, CA, USA) was introduced through the right internal jugular vein to measure pulmonary pressures. Vigilance monitor (Edwards Lifesciences, CA, USA) was connected to monitor mixed venous saturation (SvO_2) and continuous cardiac output (CCO).

Animals (n=6/group) were divided into two groups: CONTR group and ARG group (Table V.2). After baseline instrumentation, animals were preventilated (preconditioned) for 6 hours with either 21% O_2 / 79% N_2 (CONTR group) or 21% O_2 / 79% Ar (ARG group). The tidal volume of 8 ml/kg was corrected for density differences in the ARG group with the following

formula: $TV_{measured} = TV_{actual} \times \sqrt{(\rho_{test\ gas}/\rho_{100\%}O_2)}$. The density of the test gas (21% O_2 / 79% Ar) is 1.505 kg/m^3. Respiratory rate was adjusted to maintain an $ETCO_2$ of 35-45 mmHg. Every hour, lungs were recruited by increasing the PEEP temporarily from 5 cmH_2O to 20 cmH_2O. After 6 hours of preconditioning, animals were heparinized (300 IU/kg) and 1 L blood was drained to be stored with CPDA on room temperature until the next day. Lungs were cold flushed antegrade (50 ml/kg) with OCS Solution (Transmedics, Andover, USA) while ventilated with 7 ml/kg TV and 8 cmH_2O PEEP. Lungs were then inflated with the same gas mixture used during the preconditioning phase, and clamped for preservation at 25 cmH_2O. A retrograde flush was performed on the back table before storing the lungs on ice for a prolonged period of cold ischemia of 18 hours. The following day, lungs were cannulated for EVLP with the XVIVO Lung Cannula Set (XVIVO Perfusion, Göteborg, Sweden) and an 8.0 mm endotracheal tube. A postconditioning (after the ischemic insult) exposure to Ar was introduced for 4 hours during acellular (OCS solution + albumin) EVLP in a dual way (via ventilator and gas exchanger penetrating both epithelial and endothelial sides). In the ARG group, N_2 was replaced by Ar in the gas mixture of the ventilator and the gas mixture of the gas exchanger. The gas mixture settings of the gas exchanger for Ar or N_2 (3 L/min) plus CO_2 and O_2 were chosen to establish equilibrium in the oxygen content at the inflow and outflow with an $ETCO_2$ of 25-35 mmHg. Lungs were ventilated with either 12% O_2 / 88% N_2 (CONTR) or 12% O_2 / 88% Ar (ARG). In the ARG group, density corrections for the tidal volume with the test gas (12% O_2 / 88% Ar) were calculated with the same formula (test gas density 1.533 kg/m^3). Lungs were ventilated with a TV of 7 ml/kg, 7 breaths per minute respiratory rate and 5 cmH_2O PEEP. After 4 hours of postconditioning, an additional 2 hours of EVLP was performed to assess the lungs. Therefore, the acellular perfusate in the reservoir was drained and the system was primed with 1 liter of whole blood (stored on CPDA at room temperature). During this 2-hour evaluation period, lungs were ventilated with air in both groups with 8 ml/kg TV, 12 times per

minute. The perfusate was deoxygenated in the gas mixture using 10 L/min N_2 plus CO_2 and O_2 to obtain a mixed venous partial oxygen pressure with $ETCO_2$ values between 25 and 35 cmH_2O. A summary of the study protocol is visualized in Table V.2.

Table V.2 – Experimental protocol

		Baseline 1HR	***Pre-conditioning*** 6HRS	***Per-conditioning*** 18HRS	***Post-conditioning*** 4HRS	***Evaluation*** 2HRS
CONTR	Tidal Volume	8 ml/kg	8 ml/kg	Lungs stored inflated	7 ml/kg	8ml/kg
	FiO_2 ventilator	AIR	AIR	AIR	12% O_2 / 88% N_2	AIR
	Gas mixer settings	-	-	-	N_2, CO_2, O_2 matching ventilation	N_2, CO_2, O_2 deoxygenation
ARG	Tidal Volume	8 ml/kg	8 ml/kg	Lungs stored inflated	7 ml/kg	8ml/kg
	FiO_2 ventilator	AIR	21% O_2 / 79% Ar	21% O_2 / 79% Ar	12% O_2 / 88% Ar	AIR
	Gas mixer settings	-	-	-	Ar, CO_2, O_2 matching ventilation	N_2, CO_2, O_2 deoxygenation

After baseline instrumentation, animals were pre-conditioned for 6 hours with 21% O_2 and 79% N_2 (CONTR) or 79% Ar (ARG). After the preconditioning, lungs were cold flushed and stored on ice for 18 hours. Then, lungs were perfused on ex vivo lung perfusion (acellular) for 4 hours while ventilated with 12% O_2 and 88% N_2 (CONTR) or 88% Ar (ARG). Meanwhile, the perfusate was saturated with the same gas mixture but with addition of CO_2 to an $ETCO_2$ of 35-45 mmHg. At last, the saturated perfusate was drained and lungs were perfused with whole blood for an additional 2 hours on EVLP for evaluation.

FiO_2: fraction of inspired oxygen

Sampling and statistics

During the preconditioning phase in-vivo, arterial blood samples were taken hourly. Also, the following hemodynamic parameters were monitored continuously and recorded hourly: arterial blood pressure (ABP), heart rate (HR), pulmonary artery pressure (PAP), mixed venous saturation (SvO_2), continuous cardiac output measurement (CCO) and central venous pressure (CVP). After 6 hours of preconditioning, blood samples and tissue samples of liver and kidney (both fixed in formaldehyde 6%, embedded in paraffin and stained with hematoxylin-eosin) were taken to screen for potential injury due to toxicity of prolonged Ar exposure.

During postconditioning and evaluation on EVLP, physiological parameters (LAP, PAP, PVR, Ppeak) were monitored and perfusate samples were taken hourly. A 2-way ANOVA was used to compare physiological parameters and gas concentration in the perfusate during EVLP postconditioning. All data was analyzed with Graphpad 4 (GraphPad Software Inc. La Jolla CA, USA) and the level of statistical significance was set at $p<0.05$).

Lung grafts were only evaluated at the end of the evaluation period and values were compared with a Mann Whitney test. Tissue samples for histology were fixed in formaldehyde 6%, embedded in paraffin, stained with hematoxylin-eosin and scored based on injury severity by an experienced pathologist (18). Tissue samples for wet-to-dry weight ratio (W/D) calculation were taken at the end of the protocol. A bronchoalveolar lavage 30 ml saline was performed in duplicate in the right middle lobe for cytokine analysis (Cytokine Swine Magnetic 7-plex Panel for Luminex, Thermo Fisher Scientific Inc, Massachusetts, USA). Finally, the left lung was inflated at 25 cmH_2O, frozen solid in the fumes of liquid nitrogen and scanned with Siemens Somaton CT scanner (Siemens Healthcare, Erlangen, Germany). Lung mass, volume, and density were measured on the basis of the CT-scan, using imaging software (Horos^{TM}) as previously described, in which the lung is manually delineated and the number of voxels and mean density of the voxels within the volume is determined (19).

E) RESULTS

In vivo assessment

During the 6 hours of preconditioning in-vivo, animals showed a stable and comparable continuous cardiac output measurement (CCO), mixed venous saturation (SvO_2), mean pulmonary artery pressure (mPAP) and peak ventilator pressures (Ppeak) in both the CONTR and the ARG group (Figure V.7).

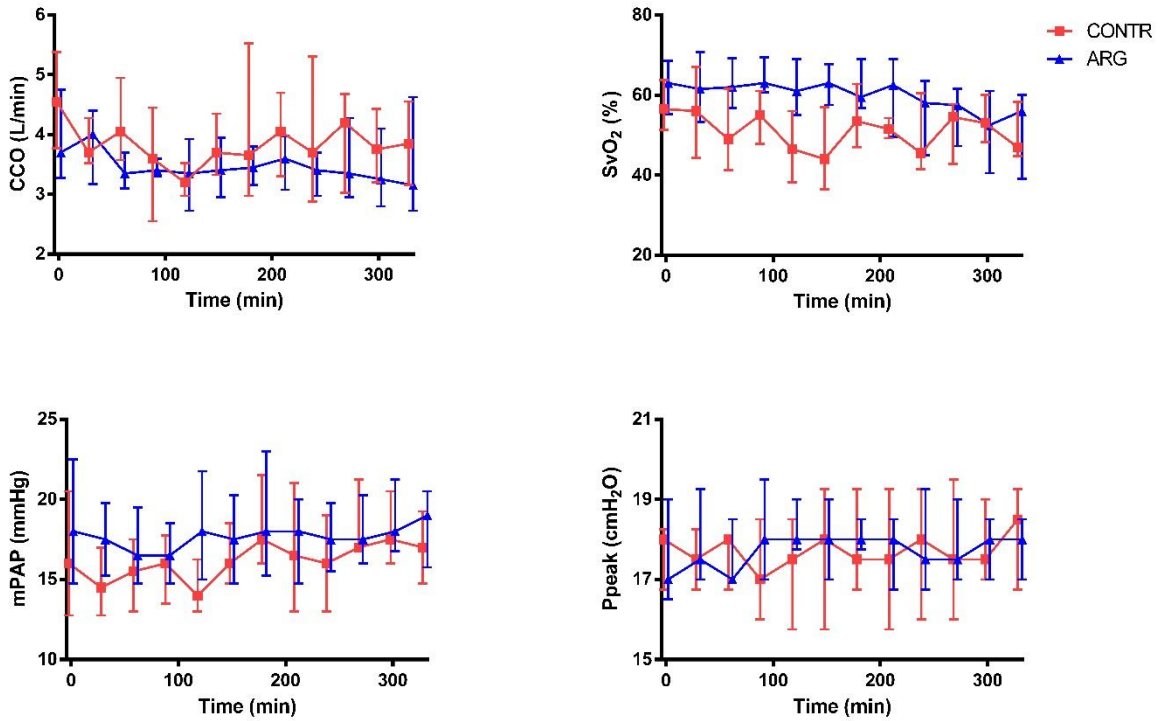

Figure V.7 – Continuous cardiac output (CCO), mixed venous saturation (SvO2), mean pulmonary artery pressures (mPAP) and peak ventilatory pressures (Ppeak) were monitored during the preventilation period. Results are depicted as median ± IQR.

After 6 hours of preconditioning, where animals in the ARG group were exposed for 6 hours to Ar through ventilation, serum analysis for kidney and liver function did not show an elevation in liver and kidney tests compared to control animals (Table V.3).

Table V.3 – Liver and kidney serum analysis.

	CONTR (n=2)	ARG (n=6)	p-value
Creatinine (mg/dL)	1.11 (1.02 - 1.20)	0.98 (0.88 - 1.05)	0,25
Ureum (mg/dL)	39.5 (33.0 - 46.0)	21.0 (18.25 - 24.0)	0,07
Alk fosf (U/L)	190.5 (153.0 - 228.0)	118.5 (99.5 - 163.5)	0,18
AST (U/L)	34.5 (29.0 - 40.0)	30.0 (25.0 - 34.0)	0,54
ALT (U/L)	38.5 (28.0 - 49.0)	50.0 (40.5 - 58.0)	0,29
Gamma GT (U/L)	59.5 (56.0 - 63.0)	50.0 (44.75 - 76.75)	0,57
LDH (U/L)	490.5 (480.0 - 501.0)	525.5 (425.5 - 575.3)	0,50
Bili (mg/dL)	<0,18	<0,18	-
CRP (mg/L)	<0,3	<0,3	-

Normal parameters are shown for both groups. Results are depicted as median (IQR). Alk fosf = alkaline phosphatase; AST = aspartate transaminase; ALT = alanine aminotransferase; Gamma GT = gamma glutamyl transpeptidase; LDH = lactate dehydrogenase; Bili = total bilirubine; CRP = C-reactive protein

Histology sections of the liver showed normal structure of liver lobules with no infiltration of inflammatory cells (Figure V.8). Histology sections of the kidney showed intact glomeruli and normal proximal and distal tubuli. There was no infiltration of inflammatory cells or deposition of necrotic epithelial cells.

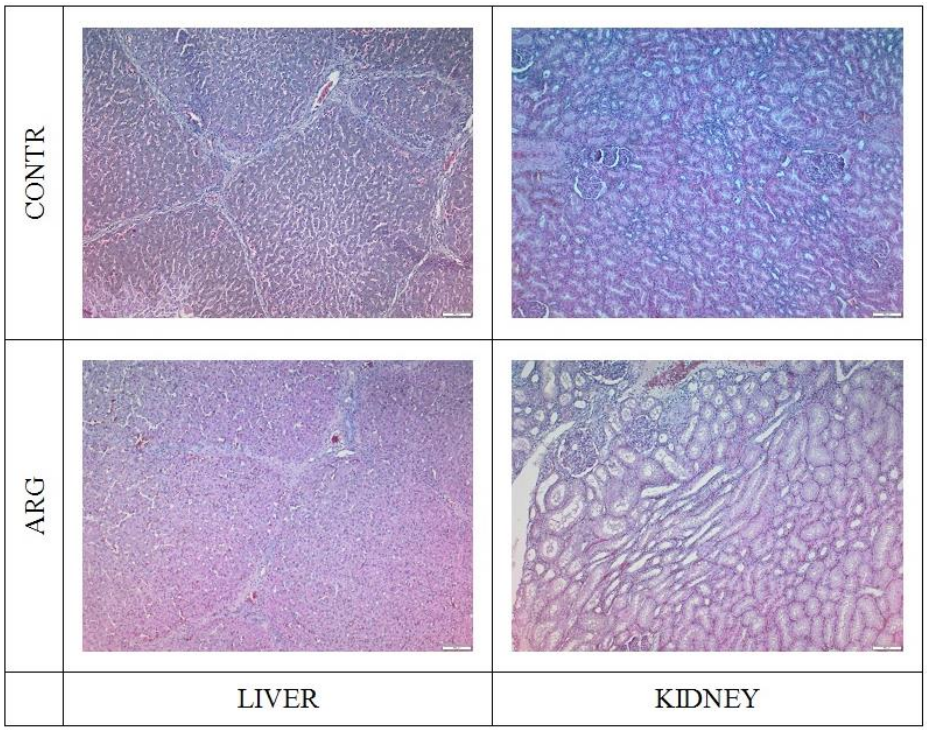

Figure V.8 – Normal histology of liver and kidney after 6 hours ventilation with Ar (ARG) or air (CONTR).

Preservation on ex-vivo lung perfusion

During the 4 hours of postconditioning on EVLP, PO_2 was stable and similar at the inflow and outflow indicating stable gas concentrations of the perfusate (Figure V.9).

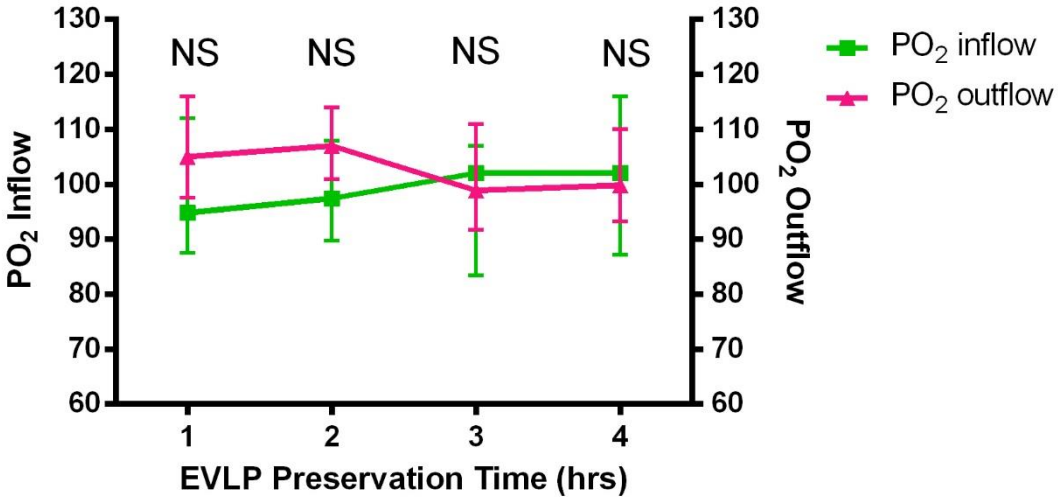

Figure V.9 – Stable PO_2 shown in out- and inflow. Results are depicted as median ± IQR and analyzed with a 2-way ANOVA (no difference between both groups).

During the postconditioning on EVLP, Ppeak and PVR remained stable in both groups and were not significantly different (Figure V.10)

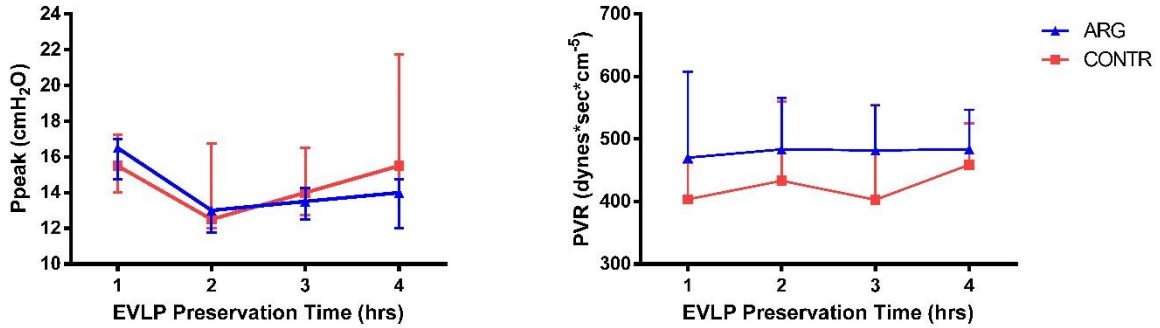

Figure V.10 – Stable airway peak pressures (Ppeak, left graph) and pulmonary vascular resistance (PVR, right graph) during postconditioning on EVLP. Data are depicted as median ± IQR and analyzed with a 2-way ANOVA (no differences between both groups).

Evaluation of graft quality

After 2 hours of perfusion with whole blood, final evaluation of physiologic parameters on EVLP did not show a significant difference in PaO_2/FiO_2, Ppeak or PVR. Also, estimation of lung edema (W/D and CT-density) did not show a significant difference between the two groups (Table V.4).

Total cell count in BAL and differential cell count (% of macrophages, neutrophils, lymphocytes) in BAL did not show a difference between both groups at the end of the evaluation on EVLP (Table V.4).

Histological sections of lung tissue showed a similar severity score (0-3) for congestion, necrosis, neutrophil influx and influx of mononuclear cells in the bronchovascular, pleuroseptal and alveolar compartment (Table V.4).

Measured cytokine levels of porcine INF-α, IFN-γ, TNF-α, IL-8, IL-1β, IL-10 and IL-4 in BAL and perfusate are shown in Table V.4. There were no significant differences between the CONTR and ARG group.

Table V.4 – Evaluation at the end of EVLP.

	CONTR	ARG	p-value
Physiology			
PaO_2/FiO_2	365 (337 - 567)	370 (325 - 507)	0.79
Ppeak (cmH_2O)	19.5 (16.5 - 24.8)	18.5 (15.8 - 22.8)	0.82
PVR ($dynes*sec*cm^{-5}$)	628 (473 - 827)	732 (655 - 926)	0.24
Lung Edema			
Wet-to-dry weight	7.4 (6.8 - 7.6)	7.5 (7.1 - 8.1)	0.47
CT density (g/L)	262 (219 - 302)	206 (161 - 311)	0.18
BAL			
Total Cell Count ($x10^9$ cells/ml)	1.75 (0.92 - 3.17)	1.20 (0.73 - 1.82)	0.47
Macrophages (% TCC)	90.9 (84.2 - 93.0)	93.9 (88.9 - 94.6)	0.39
Neutrophils (% TCC)	2.5 (1.8 - 3.7)	2.2 (1.2 - 3.8)	0.67
Lymphocytes (% TCC)	7.4 (4.7 - 12.1)	4.8 (3.9 - 6.4)	0.18
HISTOLOGY			
Mononuclear influx (ISS)	3.5 (2.8 - 6.0)	1.0 (1.0 - 3.8)	0.07
Congestion (ISS)	0.0 (0.0 - 0.1)	0.4 (0.0 - 0.5)	0.06
Necrosis (ISS)	0.0 (0.0 - 0.0)	0.2 (0.0 - 0.5)	0.18
Neutrophils (ISS)	0.1 (0.0 - 0.6)	0.5 (0.0 - 0.6)	0.57
CYTOKINES BAL			
IFN-α	0.72 (10 - 1476)	0.72 (0.71 - 0.86)	0.20
IFN-γ	0.17 (0.15 - 0.20)	0.19 (0.17 - 0.27)	0.39
TNF-α	0.03 (0.03 - 69)	61 (20 - 243)	0.11
IL-8	152 (123 - 807)	525 (214 - 1730)	0.24
IL-1β, IL-10, IL-4	Below detection limit		-
CYTOKINES PERFUSATE			
IL-1β	853 (10 - 1476)	1957 (1484 - 2352)	0.06
IL-10	25.0 (1.2 - 130.6)	81.7 (51.8 - 301.2)	0.13
IFN-α	1.4 (1.0 - 3.0)	1.8 (0.8 - 3.5)	0.62
IFN-γ	2.7 (1.1 - 4.5)	3.0 (1.3 - 11.2)	0.75
TNF-α	322 (254 - 1067)	833 (566 - 7477)	0.24
IL-8	904 (386 - 1276)	2451 (697 - 4818)	0.09
IL-4	Below detection limit		-

No differences were found between both groups (Mann-Whitney testing) at the end of EVLP at the final evaluation of graft quality in physiological parameters, lung edema estimation, BAL cell count, Injury severity scores (ISS) on histology, or cytokine levels in perfusate or BAL.
PaO_2/FiO_2 = partial oxygen pressure/fractional inspired oxygen concentration; TCC = total cell count; ISS = injury severity score; BAL = bronchoalveolar lavage

F) DISCUSSION

In this paper, we report the results of a porcine study investigating the effect of prolonged exposure to Ar on cold-ischemic lung injury with ex-vivo assessment.

Following a previous porcine pulmonary ischemia-reperfusion injury study where no effect was found by postconditioning with Ar, we designed an experimental protocol to allow a maximal exposure to Ar. The pulmonary graft was therefore exposed to Ar prior to the ischemic interval (preconditioning), during the cold ischemic injury (perconditioning) and after the ischemic interval during reperfusion on EVLP (postconditioning). The concentration of oxygen in the gas mixture for lung ventilation (FiO_2, fractional inspired oxygen concentration) was chosen, to obtain the highest concentration of Ar possible (or N_2) without inducing hypoxia to the lung tissue. During the preconditioning phase, while the animal was still alive, the FiO_2 of air was chosen (21%) and in the ARG group the N_2 in air was replaced by 79% of Ar. To allow for perconditioning, the lungs were exposed to Ar during cold preservation by inflation with the same gas mixture used as during the preconditioning phase (FiO_2 21% with 79% N_2 or Ar). The postconditioning phase during EVLP was optimized compared to the previous postconditioning protocol (7). That is: the oxygen concentration in the gas mixture of the ventilator was lowered to 12% instead of 21% to allow for a higher Ar concentration of 88% (or 88% N_2 in the CONTR group). And in addition, the perfusate was saturated with Ar via the gas exchanger to expose both the epithelial and the endothelial side of the alveolar membrane during EVLP. This saturation of the perfusion solution with Ar has also been tested in experimental kidney perfusion with improved graft function after kidney transplantation (12,13).

During 6 hours of preconditioning with Ar in-vivo, stable hemodynamic parameters were observed. Animals had normal pulmonary and systemic blood pressures, normal heart rates and normal peak ventilation pressures during the total perfusion time of 6 hours. After 6 hours of preconditioning, tissue samples from kidney and liver were taken and the pathology report did

not reveal any sign of injury to kidney or liver due to toxicity. Serum samples tested for liver and kidney function were similar between both groups. These early findings suggest that prolonged ventilation with Ar in-vivo is safe.

Maintaining a steady state of oxygen delivery and gas exchange during preservation on EVLP is a technique that has been introduced by Transmedics in the OCS^{TM} Lung protocol (20). In this set-up, partial oxygen concentration is kept stable at in- and outflow meaning no gas exchange takes place. This allows for lower gas consumption since N_2 or Ar are not continuously washed out in the gas exchanger. This technique is chosen instead of the complete wash-out of oxygen with high flow rates of N_2 or Ar, because of the scarcity and higher cost price of noble gases (21,22). During EVLP, we can monitor physiologic parameters over time while lungs are exposes to Ar and compare them with the physiologic parameters of the control group.

To fully study the effect of Ar on IRI, neutrophils will have to be present in the perfusion solution since they are the key players in the pathophysiology of ischemia-reperfusion injury (4). Therefore, after 4 hours of ex-vivo postconditioning with Ar, lungs were perfused for an additional 2 hours with whole blood containing neutrophils. With this approach, transplantation was mimicked by adding an additional two hours of perfusion with all essential blood components present. However, it can never replace the in-vivo environment and remains a surrogate for transplantation. In particular, the perfusion settings with a centrifugal pump result in a different shear stress environment and flow conditions when compared to the in-vivo pulsatile cardiac reperfusion of the lung.

At the end of this 2-hour additional whole-blood perfusion period, the pulmonary graft was evaluated by comparing physiological data, tissue samples and BAL. A median W/D of 7.4 was observed in the CONTR-group and no irreversible necrosis was seen on lung histology. In combination with an increased PVR and increased Ppeak, we can conclude that sufficient injury

was inflicted to the lung graft by our injury model to study IRI. Surprisingly, no beneficial effect was detected in physiologic parameters (Ppeak, PVR, PaO_2/FiO_2), histology, BAL cell count and cytokine measurements or lung edema (W/D and CT density) after prolonged exposure to argon in the ARG-group. We therefore concluded that attenuation of cold ischemic injury by Ar reconditioning is not beneficial in this setting. However, in other organ systems, both pre- and postconditioning by Ar have been described and the mechanisms of these effects should be further unraveled.

Evidence of an organoprotective effect in solid organ transplantation has mainly been demonstrated in kidney transplantation. Both Faure et al and Irani et al showed that saturation of the cold preservation medium with argon, resulted in a better preserved renal architecture with improved early functional recovery, graft quality and survival after kidney transplantation in their rat and pig models (12,13). Niemann et al (23) showed that hypothermia alone, in brain-dead donors also results in a reduced rate of delayed graft function among kidney transplant recipients. The effect of hypothermia or noble gases alone, has not been thoroughly investigated in solid organ transplantation so far. It might be, that an organoprotective effect of noble gases in solid organ transplantation is based on an interaction with the hypothermic protective effect. Mechanisms of a combinational therapy of hypothermia and noble gases, in combination with the interval of exposure (pre-, per-, postconditioning) should therefore be investigated further to demonstrate a protective effect in lung transplantation.

Enhancement of the neuroprotective effect of cooling by combining it with Ar exposure, has also been shown by Broad et al who demonstrated improved brain energy metabolism, faster EEG recovery and reduced cell death on TUNEL staining in a new-born piglet encephalopathy model (24). Notably, the reported functional and histopathological neuroprotective effect in other traumatic, hypoxic and ischemic brain injury models has also been demonstrated in normothermic conditions (11,25–28). A neuroprotective effect of noble gases can therefore not

be explained by an augmented protective effect of hypothermia alone. It might therefore still be possible that Ar could attenuate pulmonary injury other than (cold-) ischemic injury. For example, brain-dead donors suffer from a systemic inflammation and activation of apoptosis which could trigger the onset of severe IRI (29,30). Considering the mechanisms of brain-dead induced pulmonary damage and the potential of noble gases to promote ERK1/2 signaling through activation of MAPK in both normothermic and hypothermic conditions (31,32), further animal research, preferably using a brain-dead injury model combined with protective hypothermia and transplantation, seems appropriate.

We conclude that, although beneficial effects on ischemic injury in various other organ systems have been reported, we did not detect an improvement in pulmonary graft function after prolonged exposure to Ar in our cold-ischemic injury model with EVLP assessment. Further animal research, preferably using a brain-dead injury model, including an in-depth mechanistic investigation of the lung-protective effect of Ar, should be conducted.

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CHAPTER VI
EX-VIVO RECONDITIONING WITH MULTIPOTENT ADULT PROGENITOR CELLS

VI.A INTRAVENOUS VERSUS INTRATRACHEAL ADMINISTRATION OF MAPC®

Presented as abstract at the 2015 ESOT annual meeting Brussels (September 2015):
A. Martens, B.M. Vanaudenaerde, S.E. Verleden, R. Vos, D.E. Van Raemdonck, G.M. Verleden, C. Verfaillie, A.P. Neyrinck. Multipotent adult progenitor stem cell administration in a porcine model of ex vivo lung perfusion. Transpl Int 2015 Nov; 28:224

A) PREFACE

In the previous chapter, we have investigated the reconditioning potential of noble gases to improve lung quality prior to transplantation. In this chapter, we will focus on mesenchymal cell treatment to regulate the inflammatory process of ischemia-reperfusion injury (IRI) that can lead to severe primary graft dysfunction. In vitro and small rodent studies have shown that cellular therapy can have a beneficial immunoregulatory effect via various pathways. However, there is no data available in pre-clinical models on timing, dosing or route of administration of these cells. Therefore, we will focus on the ideal route of cellular therapy administration in the first part. We will investigate if cells should be administered in the airways or via the blood stream to tackle IRI and protect the alveolar capillary membrane.

B) ABSTRACT

Introduction - Primary graft dysfunction (PGD) compromises early outcome after lung transplantation. Others previously showed that administration of multipotent adult progenitor cells (MAPC) can be beneficial in modulating acute lung injury. Ex-vivo lung perfusion could serve as the ideal platform for cell administration and treatment evaluation. We report our first experience in administrating MAPC during EVLP in a large animal model (porcine), comparing intravascular (IV) and intratracheal (IT) administration to modulate PGD following warm ischemia-reperfusion injury.

Methods - Porcine lungs were evaluated during 6 h of EVLP after a warm ischemic interval of 90 minutes. Animals (n=6/group) were divided into 4 groups. In MAPC-IV $10*10^6$ MAPC cells were administered intravascular at the onset of EVLP, in CONTR-IV no cells were added to the perfusate. In MAPC-IT and CONTR-IT, 40 ml of PBS was instilled in the airways at onset of EVLP. In MAPC-IT only, $10*10^6$ MAPC were mixed with the PBS. At the end of EVLP, compliance, pulmonary vascular resistance, PaO_2/FiO_2, wet-to-dry weight ratio (W/D) and CT-density were evaluated. Early depletion of the perfusate due to excessive edema formation was documented. Cytokine analysis was performed on perfusate and bronchoalveolar lavage fluid.

Results – In both the IV and IT comparison, no differences were detected in physiological parameters, metabolism, graft survival or cytokine analysis. No differences were detected in W/D when MAPC cells were administered IV. However, there was less edema formation in the MAPC-IT groups compared to the CONTR-IT group (median W/D 6.6 vs 7.1).

Conclusion – We conclude that EVLP is a useful tool for administration and evaluation of cellular therapy. Only IT administration of MAPC resulted in less edema formation, however no significant differences were detected in lung physiology or the inflammatory profile of the donor lung. Future experiments should focus on timing and dosing of MAPC in the airways to detect a beneficial effect on the pathophysiology of PGD.

C) INTRODUCTION

In lung transplantation, severe primary graft dysfunction (PGD) still occurs in up to 30% of all transplanted patients with immediate impact on both short-term and long-term outcomes (1). PGD occurs within 72 hours after lung transplantation and presents itself with impaired oxygenation, lung edema and diffuse chest infiltrates (2). It is the end-result of a severe inflammatory reaction with early activation of donor macrophages, release of reactive oxygen species (ROS) and infiltration of recipient neutrophils which ultimately leads to diffuse alveolar damage (3). Clinically, only supportive treatment such as positive pressure ventilation, fluid restriction and extra-corporeal membrane oxygenation are available. To improve early outcome after lung transplantation, it is therefore crucial to modulate this inflammatory environment of donor lungs that occurs with ischemic injury during the process of organ donation, to reduce the incidence of PGD (4).

Bone marrow-derived mesenchymal cells such as the mesenchymal stem cell (MSC) and multipotent adult progenitor cell (MAPC), are increasingly being investigated in a wide spectrum of inflammatory and auto-immune disorders because of their immunoregulatory properties (5,6). Since they have limited immunogenicity, they are found to be safe and potentially efficacious (7) even in lung diseases such as acute respiratory distress syndrome that shares similar pathophysiology with PGD (8,9). These cells could therefore have the potential to modulate the rapidly evolving inflammatory cascade during reperfusion of the donor lung, based on their immunoregulatory properties. A reconditioning effect could be based on homing and differentiation of these mesenchymal cells into new structural cells. However, in the acute phase early after reperfusion, it is most likely the release of paracrine soluble factors and cell-cell interactions between these cells and structural and inflammatory cells of the lung grafts that are responsible for modulation of ischemia-reperfusion injury (IRI). For example, Mordant et

al have shown that infusion of human MSCs was associated with increased levels of human VEGF, which resulted in a decrease of porcine IL-8 expression, a marker of PGD (4).

To our knowledge, comparative data on the ideal route of administration of MAPC to the lung has not been published before. Both intratracheal (IT) and intravenous (IV) administration have been tested in separate investigations with similar outcome (4,10). In the lung, cellular treatment can thus be administered via the vasculature of the lung, or via the airways through bronchoscopy during ventilation.

As an alternative for cold static storage that leads to hypoxic lung injury, ex-vivo lung perfusion was introduced in 2001 by Stig Steen as a normothermic machine-perfusion technique with simultaneous ventilation of the donor lung in an ex-vivo setting. EVLP allows for more careful evaluation of the donor lung, but more importantly, could serve as the ideal treatment platform to improve organ quality prior to transplantation. That is, we do not compromise the donor nor the recipient in this way, and we can immediately evaluate the physiological effect of our treatment prior to transplantation. EVLP allows for administration of cells in the circulating perfusate to reach the alveolar-capillary membrane at the endothelial side, or can be used to administer cells in the airways while ventilated to reach the epithelial side.

We hypothesized that administration of MAPC at the start of EVLP would decrease IRI that leads to primary graft dysfunction. We compared the impact of MAPC administration in the airways (IT) and through perfusion (IV) vs their controls in a porcine model of reperfusion injury following warm ischemia. EVLP will be used as a platform for cellular therapy and for evaluation of the donor lung quality in an ex-vivo setting (11,12).

D) METHODS

Animals

Male domestic pigs (Topigs 20, 36-42 kg) were used and received proper care in compliance with the European Directive 2010/63/EU on the protection of animals used for scientific purposes. The Ethical Committee of the KU Leuven approved our experimental protocol (NTS P043/2014).

Animal anesthesia and baseline

Anesthesia was induced by an intramuscular injection with 5 mg/kg Zoletil 100 (Virbac s.a., Carros, France) and 3 mg/kg Xyl-M 2% (V.M.D. s.a, Arendonk, Belgium). A 20 GA peripheral intravenous catheter (Becton Dickinson Inc, Utah, USA) was inserted in the ear vein. Pancuronium bromide 2mg (Inresa Pharma, Bartenheim, Germany) and 20 µg/kg/h of fentanyl (Janssen-Cilag n.v., Beerse, Belgium) were administered for muscular relaxation and analgesia. General anesthesia was maintained with continuous intravenous infusion of 10 mg/kg/h Propofol-Lipuro 2% (B. Braun Medical n.v., Diegem, Belgium). All animals were intubated with a cuffed endotracheal tube of 7.0 mm internal diameter (Mallinckrodt^{TM} Covidien Inc, Dublin, Ireland) and ventilated in a volume-controlled mode (Aestiva 3000, GE Healthcare Europe GmbH, Little Chalfont, United Kingdom) using a tidal volume of 8 ml/kg, PEEP of 5 cmH_2O and FiO_2 of 30%. Respiratory rate was set between 20-25 breaths/min to maintain an end-tidal carbon dioxide ($ETCO_2$) that was stable between 45 and 55 mmHg. Invasive blood pressure was monitored through a 14 GA cannula inserted in the right carotid artery (Secalon-T^{TM}, Becton Dickinson Inc, New Jersey, United States).

Study groups

Animals were divided into 4 groups (n=6/group) (Figure VI.1):

- MAPC-IV group ($10*10^6$ MAPC cells were diluted in priming solution and administered IV at onset of ventilation during EVLP)
- CONTR-IV group (no cells were added)
- MAPC-IT group ($10*10^6$ MAPC cells in 40 mL Phosphate Buffered Saline (PBS) were instilled in the airways at onset of ventilation during EVLP)
- CONTR-IT group (40 mL without cells was instilled in the airways at onset of ventilation during EVLP)

In all groups, circulatory and respiratory arrest was induced by myocardial fibrillation through percutaneous puncture of the myocardium with an electrical pulse generator (current max 300 mAmp, frequency 50 Hz) and disconnection from the ventilator. Animals were then left untouched at room temperature (warm ischemic interval (WIT) of 90 min in situ).

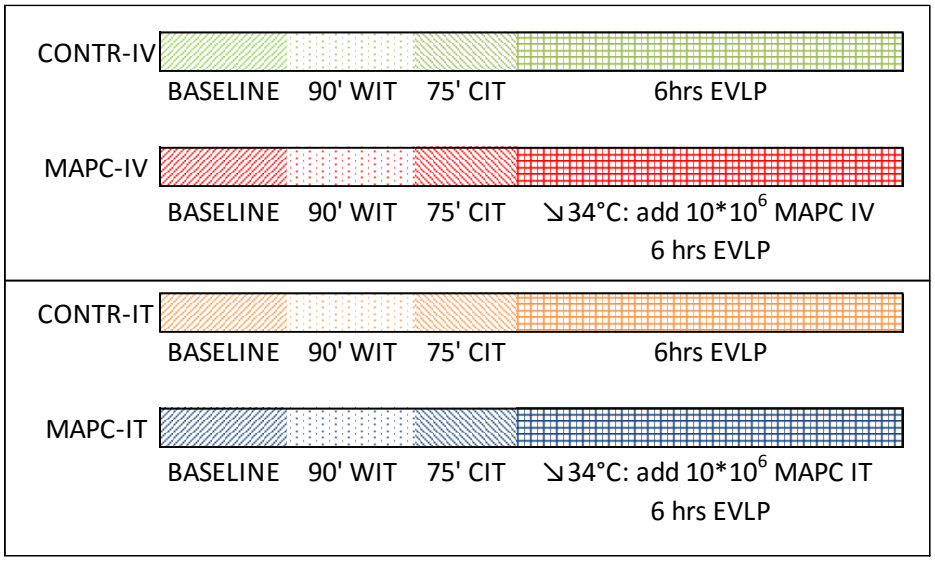

Figure VI.1 - Study groups
WIT = warm-ischemic time; CIT = cold-ischemic time

Procurement of donor lungs

A median sternotomy was performed 15 min prior to completion of the warm ischemic interval. The pericardium was opened and the pulmonary artery, superior and inferior caval veins were encircled. A purse-string was made on the right ventricular outflow tract to secure the 20 Fr

flush cannula in the pulmonary trunk (DLP Inc., Grand Rapids, Michigan USA). Following inflow occlusion through the caval veins, lungs were cold flushed (4 °C) in an antegrade way with 50 ml/kg THAM-buffered OCS™ Solution (TransMedics Inc. Andover, USA) according to the manufacturer's instructions. To optimize the flush conditions, lungs were protectively ventilated with a pressure-controlled ventilation mode using an inspiratory pressure of 15 cmH_2O and a PEEP of 8 cmH_2O.

Following the antegrade flush, the heart-lung block was excised, the heart was removed and the organs were additionally flushed in a retrograde way with a total volume of 1000 ml of OCS™ Solution. Subsequently, lungs were cannulated with the XVIVO cannulation set with closed atrium (XVIVO Perfusion AB, Göteborg, Sweden). An endotracheal tube of 8.0 mm internal diameter (Mallinckrodt Medical, Athlone, Ireland) was secured in the trachea.

Perfusate

1.5 liters of a laboratory prepared acellular solution was used. The perfusate was composed of THAM-buffered Perfadex (XVIVO Perfusion AB, Göteborg, Sweden) with 70 g/l of human albumin. Also, 15.000 IU heparin, 3.0 g glucose, 60 meq sodiumbicarbonate, 0.18 g calcium and 20 IU of insulin were added. Baseline samples of the priming solution were analyzed.

Ex-vivo lung perfusion

First, the PA cannula was de-aired and connected. Once there was flow, we also connected the outflow cannula with a vacuum effect on the reservoir (XCoatingTM, Terumo NV, Leuven, Belgium) to avoid high outflow pressures due to an air lock at the onset of EVLP. Oxygenated normothermic flow (temperature of perfusate set at 37°C) was gradually increased over a time period of one hour to a target of 40% of the estimated cardiac output, calculated as 0.1 ml/kg of the measured body weight. Left atrial pressure was kept between 3 and 5 mmHg at all times (13,14). Once the effluent reached a temperature of 34°C, volume-controlled ventilation was started with the following settings: TV of 7 ml/kg, 7 breaths/min, PEEP 5 cmH_2O and FiO_2

30%. The gas exchanger in the circuit was then provided with a mixture of oxygen, CO_2 and nitrogen to compose a mixed venous gas concentration through the inflow. Recording of blood gas results for oxygenation ((Partial oxygen pressure (PaO_2 [mmHg]) / Fractional Inspired Oxygen (FiO_2 [%])), dynamic lung compliance (Compl [ml/cmH_2O), and pulmonary vascular resistance (PVR[dynes/sec/cm^{-5}] = ((PAP[mmHg]-LAP[mmHg])*80) / Flow[L/min], were started after 1 hour of onset of EVLP until 6 hours of reperfusion. Hourly recruitment maneuvers were performed. Experiments were prematurely ended when the perfusate reservoir lost 1000ml of perfusate due to lung edema formation (defined as graft survival).

$10x10^6$ MAPC (provided by the Stem Cell Institute Leuven, Leuven, Belgium) were administered in the MAPC-IT and MAPC-IV group at the onset of ventilation. For IT instillation, the cells were diluted in 40 ml of pH-neutral PBS and distributed throughout the whole lung by bronchoscopy. In the MAPC-IV group cells were diluted in 20 ml of priming solution and slowly injected at the inflow over a period of 5 minutes. At 3 hours of EVLP 1 g of glucose and 15 meq bicarbonate were added in all experiments.

The laboratory set-up using a warm-ischemic injury model and EVLP with acellular perfusate has been previously used and is validated in our laboratory (15,16).

Tissue sampling

Lung edema of the RLL/L sample was measured using the gold standard for lung edema estimation: wet-to-dry weight ratio (W/D) (17).

At the end of the protocol tissue samples were taken and a 30 cc bronchoalveolar lavage (BAL) was performed in duplicate in the right middle lobe as previously described (18). Returned fractions were pooled and an undiluted 100 µl cytospin was stained with Diff-Quick (Dade Behring, Newark, NJ) to obtain differential cell counts. IL-1β, IL-4, IL-8, IL-10, IFN-γ, IFN-α and TNF-α were quantified in BAL supernatant with a porcine magnetic 7-plex panel (LSC001M, Thermofisher Scientific, Waltham, MA, USA).

Statistical Analysis

Each intervention group was compared with its own control (two by two comparison). MAPC-IT was compared with CONTR-IT, and MAPC-IV was compared with CONTR-IT.

Data analysis was performed with the statistical software package Graphpad Prism 4 (GraphPad Software Inc., CA, USA). All data were expressed as median ± interquartile range (IQR). In the cases where lungs could not sustain the full 6 hours of EVLP, data points recorded in the next hours after the premature end of EVLP were considered the same as the last data point available to allow comparison at all evaluation points. Statistical analysis was performed on the data at the end of EVLP (or last recorded data-point in case of early drop-out) with a Mann-Whitney test. Graft survival on EVLP was analysed with a log rank test. The level of significance was set at $p < 0.05$.

E) RESULTS

Donor Animals

Donor animals were comparable in weight between the CONTR-IT and the MAPC-IT group (p=0.39), Table VI.1, and between CONTR-IV and MAPC-IV (p=0.39), Table VI.2.

Physiological parameters during EVLP

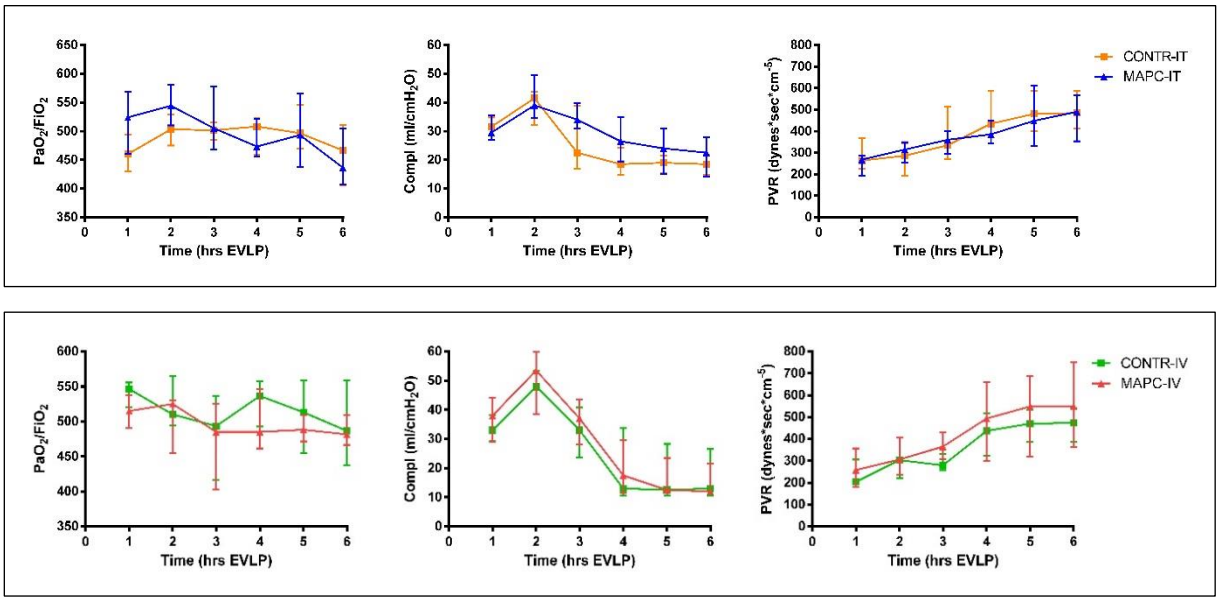

Figure VI.2 – Physiological parameters of CONTR-IT vs MAPC-IT groups in the upper panel; and of CONTR-IV vs MAPC-IV in the lower panel. Data are expressed as Median ± IQR (n=6/group).

Physiological parameters of ***IT-groups*** are depicted during the 6-hour observation on ex-vivo lung perfusion (upper panel Figure VI.2). Data registration started after a slow incremental increase in flow and temperature during the first hour. Oxygenation decreased over time in both groups and was not significantly improved in the MAPC-IT group vs CONTR-IT group, at the final evaluation moment after 6 hrs of EVLP (p=0.82). Compl initially increased due to hourly recruitment maneuvers starting at 1.5 hrs after onset of EVLP. Afterwards, Compl decreased in both IT-groups with no significant difference at the end of EVLP (p=0.39). PVR started low but slowly increased in both IT-groups over time. There was no significant improvement in

PVR in the MAPC-IT group at the end of EVLP (p=0.94) compared to CONTR-IT. Physiological parameters at the end of EVLP for the IT-groups are shown in Table VI.1.

Physiological parameters of ***IV-groups*** are depicted during the 6-hour observation on ex-vivo lung perfusion (lower panel Figure VI.2). Oxygenation also decreased over time in both IV-groups and was not significantly improved in the MAPC-IV group when looking at the final evaluation moment after 6 hrs of EVLP (p=1.0). Compl initially increased due to hourly recruitment maneuvers starting at 1.5 hrs of EVLP, similar as in IT-groups. Afterwards, Compl decreased in both IV-groups, with no significant difference at the end of EVLP (p=0.70). PVR started low, but also slowly increased over time in both IV-groups. There was no significant improvement in PVR in the MAPC-IV group at the end of EVLP (p=0.48) compared to its CONTR. Physiological parameters at the end of EVLP for the IV-groups are depicted in Table VI.2.

Graft Survival

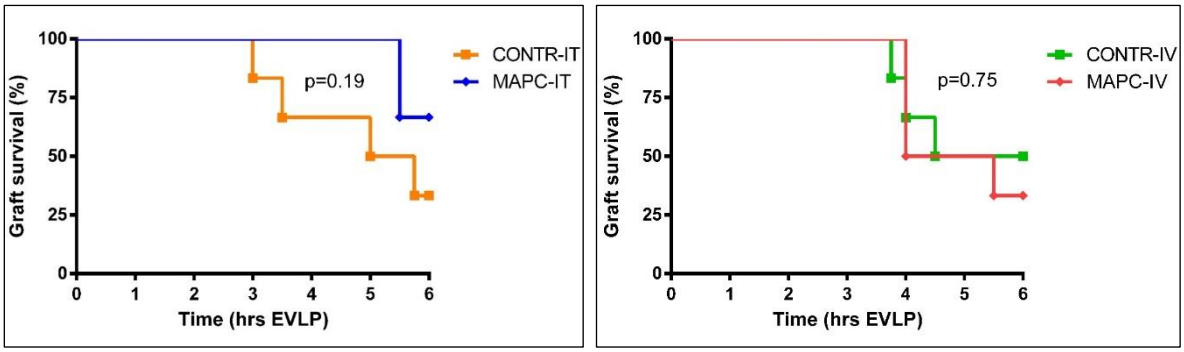

Figure VI.3 – Log-rank analysis showed a trend towards better graft survival in MAPC-IT vs CONTR-IT (left panel). There was no difference in graft survival on EVLP between the CONTR-IV and MAPC-IV groups (right panel).

Lung perfusion was stopped when more than 1000 ml of perfusate was lost due to excessive lung edema formation since the perfusate level in the reservoir became critically low. Total perfusion time was documented and depicted in Figure VI.3 to analyze graft survival on EVLP, defined by this perfusate decrease.

In the CONTR-IT group only 2 grafts were perfused for the full 6 hrs (median perfusion time 5.4 hrs), in the MAPC-IT groups only 2 grafts dropped out early at 5.5 hrs (median perfusion time 6.0 hrs). However, there was no significant difference between the 2 groups in graft survival (p=0.19).

In the CONTR-IV group, there were 3 drop-outs (median perfusion time 5.3 hrs) whereas in the MAPC-IV group there were 4 drop-outs (median perfusion time 4.8 hrs). There was no significant difference in perfusion time between both groups (p=0.75) (Figure VI.6). The development of lung edema matched with the decline in physiological parameters in all experiments.

Metabolism

Glucose consumption and lactate production are depicted in Figure VI.4 for IT-groups (upper panel) and IV-groups (lower panel). After 3 hrs of perfusion, glucose levels increased due to the addition of 1 g glucose in all experiments. At the end of EVLP, there was no significant difference between CONTR-IT vs MAPC-IT in glucose consumption (p=0.18) or lactate production (p=0.39) (Table VI.1). Also in the CONTR-IV vs MAPC-IV groups, there was no difference between both groups in glucose consumption (p=0.48) or lactate production (p=0.94) at the end of EVLP (Table VI.2).

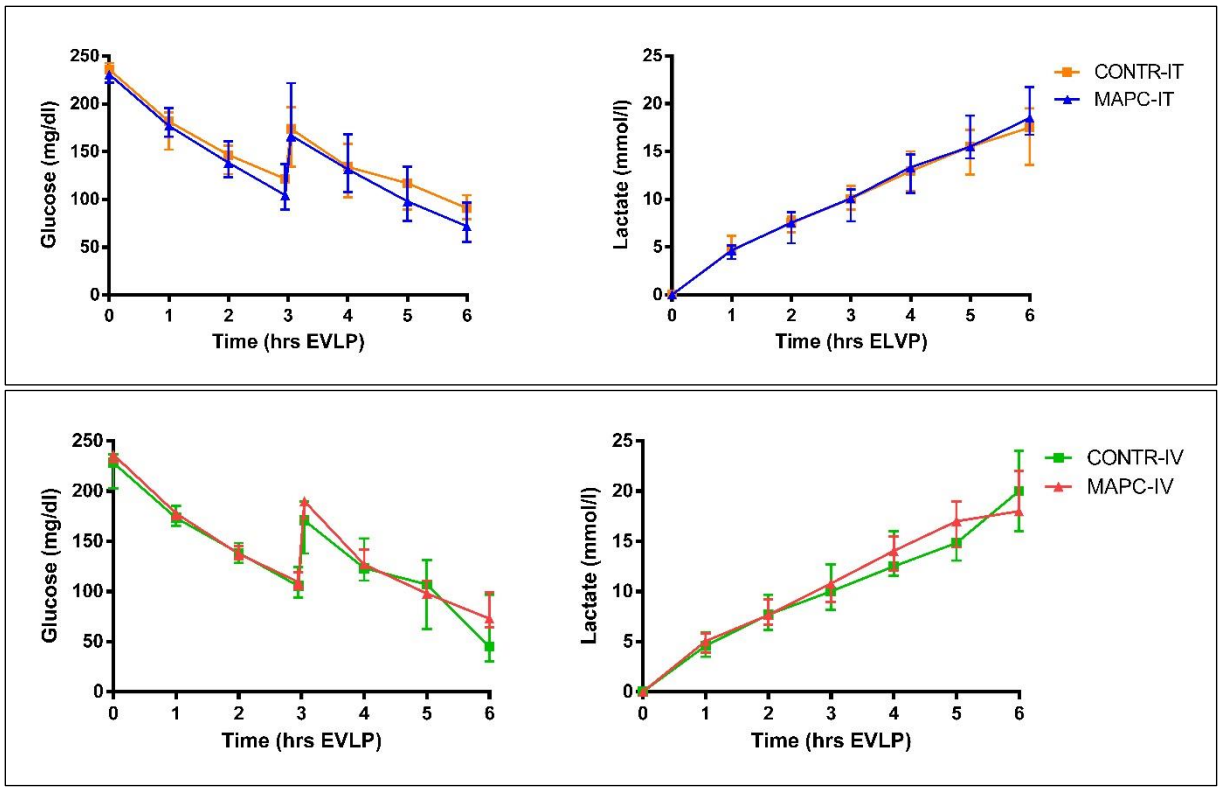

Figure VI.4 – Glucose consumption and lactate production are depicted over time for CONTR-IT vs MAPC-IT groups in the upper panel, and for CONTR-IV vs MAPC-IV groups in the lower panel. Data are expressed as Median ± IQR (n=6/group).

Estimation of Lung Edema

Lung edema estimation at the end of EVLP showed a significantly lower W/D ratio in the MAPC-IT group compared to the CONTR-IT group (p=0.03) (Figure VI.5 left panel). However, W/D ratio did not differ between the CONTR-IV and MAPC-IV group (p=0.70) (Figure VI.5 right panel).

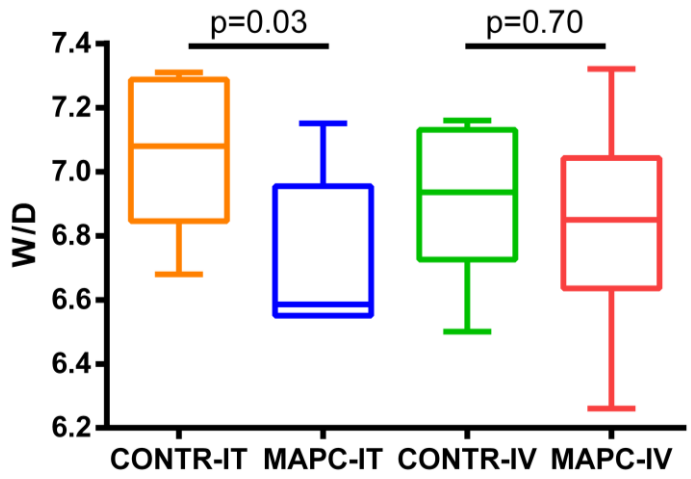

Figure VI.5 W/D ratio was significantly reduced in the MAPC-IT vs CONTR-IT group, however was not reduced in the MAPC-IV vs CONTR-IV group. Data are depicted as boxplot and compared with a Mann-Whitney test.

Cytokine Analysis

Results of the Multiplex ELISA analysis of the BAL fluid of the CONTR-IT vs MAPC-IT is shown in Table VI.1. IL-8 and IL-1β were above and below detection limit, respectively. There were no significant differences between the IT-groups in IL-10 (p=0.31), IFN-α (p=0.82), IFN-γ (p=0.48), TNF-α (p=0.39) or IL-4 (p=0.48).

Table VI.1 – Donor animal characteristics, EVLP parameters at the end of EVLP, ELISA-analysis of BAL fluid and metabolic parameters at the end of EVLP compared between CONTR-IT and MAPC-IT group.

		CONTR-IT	MAPC-IT	p-value
Donor animal	**Weight (kg)**	41.6 (41.3 - 52.1)	43.3 (40.8 - 45.4)	0.39
EVLP Parameters	**PaO_2/FiO_2**	468 (400 - 518)	437 (400 - 507)	0.81
End EVLP	**Compliance (ml/cmH_2O)**	18.5 (13.5 - 22.5)	22.5 (13.5 - 29.0)	0.39
	PVR (dynes*sec*cm^{-5})	485 (390 - 597)	491 (328 - 584)	0.94
	Survival (hours)	5.4 (3.3 - 6.0)	6.0 (5.5 - 6.0)	0.24
BAL	**IL-10**	1.1 (0.6 - 2.8)	2.4 (0.7 - 7.3)	0.31
	IFN-α	0.4 (0.3 - 0.7)	0.4 (0.3 - 3.0)	0.82
	IFN-γ	0.19 (0.16 - 0.26)	0.20 (0.16 - 0.34)	0.48
	TNF-α	226 (158 - 545)	275 (235 - 441)	0.39
	IL-4	0.22 (0.21 - 0.24)	0.24 (0.21 - 0.26)	0.48
	IL-8	*above detection limit*	*above detection limit*	
	IL-1β	*below detection limit*	*below detection limit*	
Metabolism	**Lactate (mmol/l)**	17.5 (12.2 - 20.0)	18.5 (16.5 - 22.5)	0.39
	Glucose (mg/dl)	91 (76 - 111)	72 (52 - 100)	0.18
Lung edema	**W/D**	7.1 (6.8 - 7.3)	6.6 (6.6 - 7.0)	0.03*

Data are compared with a Mann-Whitney test and are depicted as median (25% QI - 75% QI).

Results of the Multiplex ELISA analysis of the BAL fluid compared between CONTR-IV vs MAPC-IV, are shown in Table VI.2. IL-1β was below the detection limit. There were no significant differences between the IV-groups in IL-10 (p=0.94), IFN-α (p=0.39), IFN-γ (p=0.48), TNF-α (p=0.59), IL-4 (p=0.94) or IL-8 (p=1.00).

Table VI.2 - Donor animal characteristics, EVLP parameters at the end of EVLP, ELISA-analysis of BAL fluid and metabolic parameters at the end of EVLP, compared between the CONTR-IV and MAPC-IV group.

		CONTR-IV	MAPC-IV	p-value
Donor animal	**Weight (kg)**	43.0 (39.0 - 45.9)	40.8 (37.4 - 44.6)	0.39
EVLP Parameters End EVLP	**PaO_2/FiO_2**	487 (435 - 462)	482 (460 - 522)	1.00
	Compliance (ml/cmH_2O)	13 (10 - 30)	12 (11 - 22)	0.70
	PVR (dynes*sec*cm^{-5})	474 (344 - 546)	549 (344 - 774)	0.48
	Survival (hours)	5.3 (3.9 - 6)	4.8 (4.0 - 6.0)	1.00
BAL	**IL-10**	2.2 (1.0 - 9.1)	2.2 (0.6 - 10.6)	0.94
	IFN-α	0.4 (0.3 - 0.8)	0.4 (0.4 - 0.7)	0.39
	IFN-γ	0.2 (0.2 - 0.3)	0.3 (0.2 - 0.6)	0.48
	TNF-α	262 (149 - 466)	348 (206 - 700)	0.59
	IL-4	0.2 (0.2 - 0.3)	0.2 (0.2 - 0.3)	0.94
	IL-8	680 (93 - 1193)	662 (146 - 1172)	1.00
	IL-1β	*below detection limit*	*below detection limit*	
Metabolism	**Lactate (mmol/l)**	16.0 (13.3 - 22.0)	16.5 (13.1 - 20.0)	0.94
	Glucose (mg/dl)	109 (38 - 127)	112 (69 - 137)	0.48
Lung edema	**W/D**	6.9 (6.7 - 7.1)	6.9 (6.5 - 7.1)	0.70

Data are compared using a Mann-Whitney test and are depicted as median (25% QI -75% QI).

F) DISCUSSION

In this study, we have demonstrated that administration of cellular therapy during EVLP in the airways (IT) (not when administered via the vasculature (IV)) can reduce lung edema formation in a porcine model with warm-ischemic lung injury. Graft function after administrating $10*10^6$ MAPC in the airways (IT) or in the vasculature (IV) of the lung, during ex- vivo lung perfusion, was compared with physiological and immunological assessment with EVLP.

With these experiments, where we administered cellular therapy in both airways and in the pulmonary artery, we confirm that EVLP is an excellent platform for ex-vivo organ treatment prior to transplantation (12). However, up to now, there is no consensus on how to deliver cellular therapy to the lungs, either via the airways or via the blood stream. If stem cells are injected intravenously, they are initially trapped inside the lungs due to their large cell size (15-19 micrometre) (19). This phenomenon is often referred to as the pulmonary first-pass effect (20). Although this effect might impede a potential therapeutic effect in other organ systems after intravenous administration, this effect is beneficial for the lungs since most are trapped in the lung in the acute setting. Nonetheless, there is an increased risk of pulmonary hypertension due to micro-thrombi formation which can lead to increased PVR and hydrostatic pulmonary oedema. Beneficial effects of stem cell administration to reduce pulmonary injury has been described with both delivery in the airways or in the vasculature of the lungs (4,11,21). However, since primary graft dysfunction starts with diffuse alveolar damage and epithelial denudation that results in capillary leakage, we hypothesized that instillation of stem cells at the epithelial site could be most beneficial (22). Also, we are the first to investigate the effect of MAPC in a controlled animal study of pulmonary IRI.

Less edema formation was detected in the MAPC-IT group based on a lower W/D at the end of EVLP. This effect was not seen when stem cells were administered IV. Therefore, we conclude that a beneficial effect of cellular treatment has the greatest potential when cells are

administered in the airways. Unfortunately, this reduction in lung edema in the MAPC-IT group was not translated into improved physiological parameters compared to its CONTR-IT group. However, until today there is no consensus on which parameters are most reliable for the evaluation of pulmonary graft function ex-vivo. More and more, lung compliance is chosen over pulmonary vascular resistance and oxygenation capacity since it is the first parameter that changes with the development of lung edema (23). Also in our experiments we see that oxygenation remains stable longer in comparison with lung compliance (and pulmonary vascular resistance). Prediction of graft quality after lung transplantation based on physiological parameters remains therefore difficult. So although no differences were found in physiologic parameters in our experiments, there was less edema formation in the MAPC-IT group. Therefore, MAPC cells delivered in the airways did effect the end-result of ischemia-reperfusion injury: accumulation of alveolar edema. A transplant model with evaluation of the graft quality after whole-blood perfusion in the recipient could therefore shed more light on the true effect of mesenchymal cell administration.

In addition to a lower W/D ratio, grafts that received multipotent adult progenitor cells in the airways tend to drop-out later on EVLP (median perfusion time 6.0 hours (5.5 – 6.0 IQR)) than pulmonary grafts in all other groups (median perfusion time 5.25 hours (4.0 – 6.0 IQR)). Although this effect was statistically not significant (p=0.19), we did feel that this was an important tendency for future experiments.

In solid organ transplantation, cellular ex-vivo treatment focuses on mesenchymal cells. Both the mesenchymal stem cell (MSC) and the multipotent adult progenitor cell (MAPC) are bone-marrow derived mesenchymal cells with similar immunoregulatory properties (24,25). The MSC has already been investigated in ischemia-reperfusion injury models of kidney and liver. In both these models, there was a better preserved histological architecture, and enhanced graft function after transplantation when stem cells were administered to the graft (26,27). Also in

lung transplantation, pre-clinical data on graft enhancement has been published now (4). Preliminary data with similar results in pulmonary grafts have been published for the MAPC (10), however comparative pre-clinical data is lacking. Therefore, we believe that more research needs to be conducted before this therapy can be safely introduced in current clinical solid organ transplant programs.

Although an effect was detected by other research groups, we did not detect an alteration in graft function based on physiologic parameters and cytokine expression profiles after administration of 10 million cells in lungs that were injured by warm ischemia. However, some limitations in this study might have led to false-negative results. First, timing and dosing of cellular treatment has not been widely investigated. In mice studies, 0.5 to 2 million cells are usually administered however, these doses correspond to 100 to 200 million cells/kg which is widely irrelevant for clinical practice. In our study design, we administered a dose of 20 million cells / (kg lung weight). We based the dosing on the lung weight rather than on body weight, since cells were administered to the lungs only in an ex-vivo setting. Other research groups in solid organ transplantation (liver, kidney) showed that an intermediate dose of 3.5 to 4.5 million cells / (kg total body weight) can reduce graft failure (26,27). This dose is based on total body weight, even though grafts are perfused ex vivo (4). If we were to calculate our administered dose on total body weight (mean 42.5kg), we can conclude that the used dose in this protocol (0.24 million/kg) might have been insufficient to induce a physiological improvement.

Secondly, 90 minutes of warm ischemia after induction of ventricular fibrillation and ventilator switch-off was the chosen injury model in these experiments (16). Warm-ischemic injury models have been used by many research groups in the past, and also our research group has a lot of experience with this injury model (28). Different injury models to study ischemia-reperfusion injury have been used in large animal models (29,30) and it is known that the mode of dying has a large impact on graft function (31). Therefore, also other types of injury models,

especially brain-dead, should be investigated to detect the impact of cellular therapy on the inflammatory cascade after ischemia-reperfusion injury (32). Thirdly, stem cells were administered after the injury was introduced (postconditioning). One could defend to introduce treatment earlier in order to prevent ischemia-reperfusion injury from happening (preconditioning). Wittwer et al showed that when MSCs were administered endobronchially prior to the preservation period (preconditioning the donor), dynamic lung compliance was better preserved compared to controls (21). We do have to keep in mind that this can be a logistical challenge in the timeframe of organ procurement and preservation, and that ex-vivo lung perfusion still gives us the ex-vivo advantage of not potentially harming the donor, nor the recipient.

In conclusion, we can state that administration of 10 million MAPC intravenously, in a warm-ischemic injured porcine lung, did not result in improvement of physiological parameters, lung edema formation, cytokine expression, or graft survival. Also after intratracheal administration of MAPC cells, no beneficial effect on lung physiology, metabolism or inflammation was detected. However, there was a tendency for longer graft survival on ex-vivo lung perfusion and there was a reduction in alveolar edema in the MAPC-IT group. Future experiments should explore the mechanisms of MAPC treatment, administered in the airways, that potentially lead to reduced IRI severity and incidence.

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CHAPTER VI
EX-VIVO RECONDITIONING WITH MULTIPOTENT ADULT PROGENITOR CELLS

VI.B IMMUNOREGULATORY EFFECT OF MAPC® DURING EVLP

Submitted for publication:

Martens A, Ordies S, Vanaudenaerde BM, Verleden SE, Vos R, Van Raemdonck D, Verleden GE, Roobrouck V, Claes S, Schols D, Verbeken EK, Neyrinck AP. Immunoregulatory effects of multipotent adult progenitor cells in a porcine ex-vivo lung perfusion model

A) PREFACE

In the previous chapter, we investigated the ideal route of cell treatment (intratracheal or intravenous) in a pre-clinical porcine model. We concluded that administration of cells in the airways is most efficient based on less lung edema development. This matches with our hypothesis that the alveolar-capillary membrane is best reached from the epithelial side (the airways) since the injurious process of ischemia-reperfusion injury (IRI) starts with interstitial edema and denudation of the epithelial membrane. In the final chapter of my thesis, we will focus on the mechanisms of IRI reduction after multipotent adult progenitor cell (MAPC®) administration in the airways. We hypothesize that instillation of MAPC cells in the airways can have an immunoregulatory effect in the process leading to ischemia-reperfusion injury.

B) ABSTRACT

Introduction - Primary graft dysfunction (PGD) is considered to be the end result of an inflammatory response targeting the new lung allograft after transplant. Previous research has indicated that MAPC cell therapy might attenuate this injury by its paracrine effects on the pro-/anti-inflammatory balance. This study aims to investigate the immunoregulatory capacities of MAPC cells in PGD when administered in the airways.

Methods - Lungs of domestic pigs (n=6/group) were subjected to 90 min of warm ischemia. Lungs were cold flushed, cannulated on ice and placed on EVLP for 6 hours. At the start of EVLP, 40 ml of an albumin-plasmalyte mixture was instilled in the airways (CONTR-group). In the MAPC cell group, 150 million MAPC cells (ReGenesys/Athersys, Cleveland, USA) were added to this mixture. At the end of EVLP, a physiological evaluation (pulmonary vascular resistance, lung compliance, PaO_2/FiO_2), wet-to-dry weight ratio (W/D) sampling and a multiplex-analysis of bronchoalveolar lavage (BAL) (2x30 ml) was performed.

Results - Pulmonary vascular resistance, lung compliance, PaO_2/FiO_2 and W/D were not statistically different at the end of EVLP between both groups. BAL neutrophilia was significantly reduced in the MAPC cell group. Moreover, there was a significant decrease in TNF-α, IL-1β and IFN-γ in the BAL, but not in IFN-α; whereas IL-4, IL-10 and IL-8 were below the detection limit.

Conclusion - Although no physiologic effect of MAPC cell distribution in the airways was detected during EVLP, we observed a reduction in pro-inflammatory cytokines and neutrophils in BAL in the MAPC cell group. This effect on the innate immune system might play an important role in critically modifying the process of PGD after transplantation. Further experiments will have to elucidate the immunoregulatory effect of MAPC cell administration on graft function after transplantation.

C) INTRODUCTION

Anno 2016, lung transplantation has grown into a successful treatment option for patients with end-stage pulmonary disease. However, severe primary graft dysfunction (PGD) still occurs in up to 30% of transplanted patients. And although there is a low mortality of PGD due to successful supportive therapy nowadays, patients with severe PGD have poorer long-term outcome and a higher risk for developing chronic lung allograft dysfunction (CLAD). PGD is the end-result of ischemia-reperfusion injury (IRI) attacking the integrity of the capillary-alveolar membrane leading to pulmonary edema and impaired oxygenation (1,2). It is based on an inflammatory cascade that is triggered by hypoxic stress and activation of donor macrophages which attracts many recipient neutrophils to the donor lung upon reperfusion in the recipient chest. Bone-marrow derived mesenchymal cells possess immunoregulatory capabilities and could therefore be of particular interest to attenuate PGD. They are already found to be a successful treatment option for patients suffering from acute-respiratory distress syndrome, which shares a similar inflammatory pathophysiology with PGD (3–5). More specifically, pilot studies show that they can reduce IRI inherent to solid organ transplantation in the lung (6–8), but also in other organ systems (9,10). Here, their beneficial effects result from paracrine mechanisms and cell-cell interaction rather than engraftment and repair of diseased tissue (11). An altered inflammatory balance, with a decrease in pro-inflammatory and increase in anti-inflammatory cytokines, is observed (6). In hypoxic conditions, such as ischemic injury models, secretion of growth factors such as VEGF and ANG-1 can stimulate angiogenesis and tissue repair (8,12). Mordant et al recently published their results on MSC administration to a porcine donor lung on EVLP which resulted in a reduction of IL-8, however, there was no effect on physiological parameters detected (13). The role in a reduction of inflammatory cytokines in an acellular ex-vivo perfusion set-up therefore still has to be unraveled.

There are two types of bone-marrow derived cells that are well characterized and could be good candidates for immunoregulation of the IRI pathophysiology: the mesenchymal stem cell (MSC) and MAPC cells (14). Both have similar immunoregulatory capabilities, but are characterized as individual cell types and adopt different phenotypes under certain culture conditions (15). One of the most advantageous characteristics of the MAPC cells is their large proliferation capacity and low senescence. Therefore, large batches of stem cells can be produced from one healthy donor (16,17). A clinical grade MAPC product (MultiStem®, Athersys, Cleveland, USA) has been developed for Phase I and II clinical testing. The MultiStem clinical grade product is based on MAPC cell isolation and expansion protocols under good manufacturing practice conditions (18,19). Our previous research indicates that intratracheal administration of cells holds the greatest potential to reduce IRI based on a reduction in lung edema formation. However, after administration of 10 million MAPC cells in the airways, a physiological improvement of lung function was not detected nor was there an alteration in the inflammatory environment of the lung tissue. Therefore, in this study we increased the dosing regimen to an intermediate dose of 3.75 million/kg in order to investigate a physiological improvement of lung function and to study this mechanism based on immunoregulation. Also for other solid organ transplant research, this intermediate dosing regimen of 3.5 - 4.5 million cells/kg has led to an improved graft function after transplantation (4,9,10).

In order to administer these cells, ex-vivo lung perfusion (EVLP) has been put forward as a treatment platform of ex-vivo organ reconditioning. EVLP is a preservation technique of donor lungs in normothermic conditions, allowing for continuous evaluation of the donor lung by perfusing and ventilating the graft in an ex-vivo lung perfusion device. EVLP allows for assessment of higher risk donor lungs and might serve as the ideal platform for active treatment

since donor and recipient remain unharmed, and the treatment effect can be immediately evaluated by interpretation of the physiological parameters (20,21).

The immunoregulatory properties of MAPC cells have not been widely studied in pre-clinical large animal models and most preliminary theories of working mechanisms are based on in-vitro and rodent models. Therefore, in this large animal study, we aim to investigate if MAPC cell delivery in the airways during EVLP (i.e. postconditioning), can modulate the inflammatory process linked to IRI by immunoregulatory effects.

D) METHODS

This experimental study was performed in compliance with the European Directive 2010/63/EU on the protection of animals used for scientific purposes. The principles of laboratory animal care published by the National Institute of Health Volume 25, No. 28 (revised 1996) were followed. Local ethics approval was obtained at the research institute (NTS P043/2014).

Donor procedure

Domestic pigs Topig 20 (mean 40.8 kg) were divided into 2 groups (n=6/group). Animals were anesthetized with an intramuscular injection of 5 mg/kg Zoletil 100 (Virbac, Carros, France) and 3 mg/kg Xyl-M 2% (VMD, Arendonk, Belgium). Anaesthesia was maintained using 10 mg/kg/h propofol, 20 µg/kg/h fentanyl and intermittent boli of pancuronium 2mg for muscle relaxation. Animals were intubated with a 7.0 mm endotracheal tube and ventilated (Aestiva 3000; GE Healthcare Europe GmbH, Little Chalfont, UK) with a tidal volume (TV) of 8 ml/kg, positive end-expiratory pressure (PEEP) of 5 cmH_2O and FiO_2 of 30%. Respiratory rate (RR) was adjusted to the end-tidal carbon dioxide ($ETCO_2$) (45-55 mmHg). Blood pressure was monitored invasively in the right carotid artery. All animals died of cardiac arrest which was induced by direct electrical stimulation of the myocardium with an electrical pulse generator that led to ventricular fibrillation. Animals were disconnected from the ventilator when cardiac arrest was induced. Prior to cardiac arrest, all animals were heparinized with 300 IU/kg.

Following cardiac arrest in the donor, grafts were left untouched in the deceased donor for 90 minutes after which they were flushed antegradely with 50 ml/kg cold thromethamol-buffered OCS Solution (Transmedics, Andover, USA). The heart-lung block was excised and a retrograde flush (1L thromethamol-buffered OCS solution) was performed at the back table. Lungs were instrumented on ice for a short period of time (mean 64.4 min) while the XVIVO (Göteborg, Sweden) cannulas were secured in the pulmonary artery and atrial cuff. An 8.0-mm

ET tube was secured in the trachea. The donor procedure was performed as previously described (22).

Multipotent adult progenitor cell preparation

Human MAPC cells were isolated by Athersys/Regenesys (Athersys, Cleveland, USA; Regenesys, Heverlee, Belgium) from bone marrow of healthy volunteers. Isolation and cultivation of the MAPC cells were based on previously published protocols (19,23). The Quantum Cell Expansion System (Terumo BCT, Lakewood, CO, USA) was used for ex-vivo expansion of large batches of MAPC. All cell batches were subjected to several quality control assays to test if all MAPC cell criteria were met. First of all, cell quality is assessed by measuring viability and plating efficiency. Secondly, cells are identified using qPCR and flow cytometry to test both negative and positive markers (24). A tube formation assay is done to define the proangiogenic activity (25), a CFSE assay (26) is performed for evaluating the immunoregulatory capacity.

Ex-vivo lung perfusion

Lungs are perfused ex-vivo with an acellular albumin containing dextran solution. The production of the perfusate and technique of EVLP are performed as described previously (27). After a 1-hour rewarming period and slow increase of the flow to 40% of the estimated cardiac output (calculated as 100 ml/kg) lungs were further perfused and evaluated for 6 hours in total. Once the outflow temperature reaches 34°C, ventilation was started with 7 ml/kg tidal volume for 7 breaths/min with 5 cmH_2O PEEP. In the CONTR-group, 40 ml of acellular albumin (2.5%) – plasmalyte mixture was distributed with a bronchoscope throughout the lung when ventilation was started. In the MAPC cell group, 150 million MAPC cells were added to this mixture. The study protocol is outlined in Figure VI.8. Cells were thawed, PBS washed and diluted in 40 ml of the albumin - plasmalyte mixture after confirming that the donor lungs were free of adhesions

or infiltrates. During 6 hours of EVLP, dynamic lung compliance (Compl), oxygenation (PaO_2/FiO_2) and pulmonary vascular resistance (PVR) was recorded hourly.

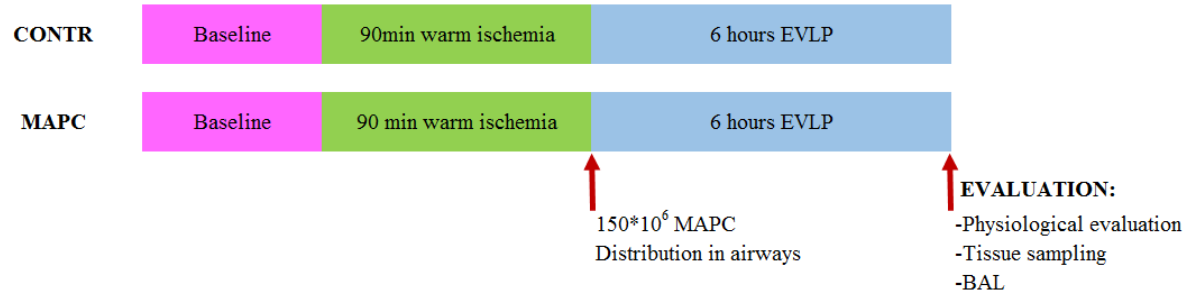

Figure VI.8 – Study protocol with n=6/group.

Tissue sampling

At the end of the experiment, tissue samples were taken for histological evaluation and wet-to-dry-weight (W/D) ratio calculation (after 48 hrs in the oven at 80°C). Pathology samples are scored by an experienced pathologist (EV) for congestion, neutrophil influx and necrosis. Bronchoalveolar lavage (BAL) with 2 times 30 cc saline 0.9% was performed in the right middle lobe. Pooled fractions were returned and a cytospin (100µl) was stained with Diff-Quick (Dade Behring, Newark, NJ) to perform total and differential cell counts. The BAL supernatant was analyzed with a porcine multiplex ELISA kit for IL-1β, IL-4, IL-8, IL-10, IFN-γ, IFN-α and TNF-α according to the manufacturer's protocol (Thermo Fisher Scientific Inc, Massachusetts, USA). The left lung was inflated at 25 cmH_2O, frozen solid in the fumes of liquid nitrogen and scanned with Siemens Somaton CT scanner. Lung mass, volume, and density were measured on the basis of the CT-scan, using imaging software (Horos®) in which the lung is manually delineated and the number of voxels and mean density of the voxels within the volume is determined (28).

Statistical analysis

All data are expressed as median with IQ range (IQR) when depicting physiological variables in time or as a scatter plot with median and IQR when comparing variables at the end of the experiment (GraphPad Prism 4, GraphPad Software Inc, La Jolla, USA).

Mann-Whitney tests were conducted in GraphPad to compare data at the end of EVLP. We analyzed end-experimental parameters only to dichotomize between acceptable and non-acceptable lungs. Baseline parameters of the donor animals are described as median (25% QI – 75% QI) and are analyzed with the same statistical test. The level of statistical significance was set at $p<0.05$.

E) RESULTS

Baseline

Baseline animal parameters and the perfusate are similar between both groups (Table VI.3).

Table VI.3 – Baseline animal parameters and perfusate composition

	CONTR	MAPC	p-value
Baseline animal			
Weight (kg)	40.3 (38.8 - 41.2)	42.3 (40.2 - 43.1)	0.10
Vt (mL/kg)	7.9 (7.8 - 8.0)	7.9 (7.8 - 7.9)	0.85
HR (bpm)	88 (73 - 118)	107 (94 - 148)	0.17
MAP (mmHg)	102 (90 - 113)	96 (77 - 106)	0.42
Peak AwP (cmH_2O)	19.5 (18.8 - 21.3)	18.5 (17.5 - 19.3)	0.16
PaO_2/FiO_2 (mmHg)	413 (399 - 455)	405 (376 - 421)	0.37
WBC (10^9/L)	19.8 (14.3 - 24.7)	18.5 (15.2 - 21.2)	0.62
Neutrophils (%)	42.5 (32.3 - 51.0)	46.0 (37.5 - 52.5)	0.68
Perfusate composition			
Albumine (g/L)	65.5 (64.0 - 66.9)	66.8 (63.5 - 68.8)	0.47
Osmolaliteit (mmol/kg H_2O)	318 (614 - 320)	318 (315 - 319)	0.85
Na (mmol/L)	154 (154 - 156)	155 (153 - 156)	0.79
K (mmol/L)	3.5 (3.5 - 3.6)	3.5 (3.5 - 3.6)	0.51
Cl (mmol/L)	105 (104 - 106)	105 (104 - 105)	0.29
Bicarbonaat (mmol/L)	31.2 (29.6 -31.9)	32.6 (30.6 - 33.1)	0.16
Ca (mmol/L)	0.57 (0.55 - 0.57)	0.53 (0.52 - 0.56)	0.10
Glucose (mg/dL)	233 (230 - 239)	236 (229 - 248)	0.98

Comparable animal weight and baseline hemodynamic parameters. No signs of systemic inflammation (normal WBC count, normal neutrophil count, normal temperature). Perfusate composition is similar between both groups for albumin, osmolality, glucose and electrolytes. Vt: Tidal Volume; HR: heart rate; MAP: mean arterial pressure; Peak AwP: peak airway pressure; PaO_2/FiO_2: partial oxygen pressure/fractional inspired oxygen concentration; WBC: white blood cell count; Na: sodium; K: potassium; Cl: chloride; Ca: calcium

Physiological assessment

Physiological parameters PVR, Compl, and PaO_2/FiO_2 are depicted over time (hrs on EVLP) in panel A-C of Figure VI.9. All data points are depicted as median +/- IQR.

Physiologic parameters at the end of EVLP, are depicted in panel D-F. No statistical differences were detected in PVR (p=0.68), Compl (p=0.22) or PaO_2/FiO_2 (p=0.13) between the two groups.

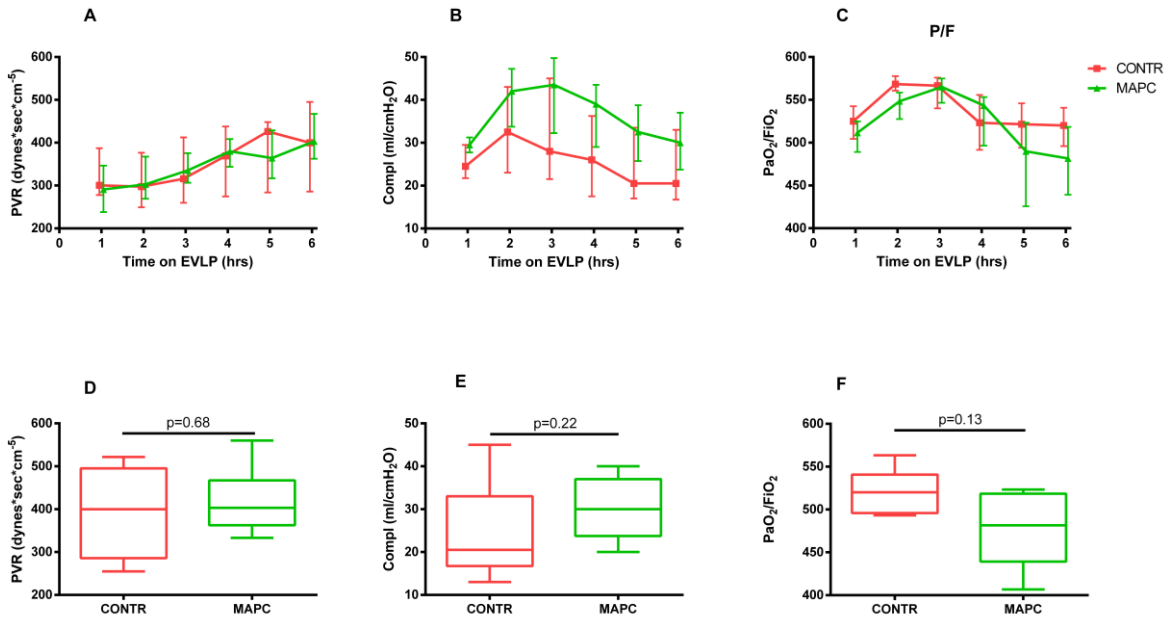

Figure VI.9 – Monitoring of PVR (Panel A), Compl (Panel B) and PaO_2/FiO_2 (Panel C) during 6 hrs of EVLP. The final assessment at the end of EVLP did not show any statistical difference between both groups for PVR (Panel D), Compl (Panel E) or PaO_2/FiO_2 (Panel F).

Lung edema estimation

W/D of the tissue sample of the right lower lobe and CT density calculation of the left inflated frozen lung did not reveal a statistical difference between both groups (Figure VI.10). Administration of 150 million stem cells did not result in attenuation of lung edema formation in the warm-ischemic injured porcine lungs.

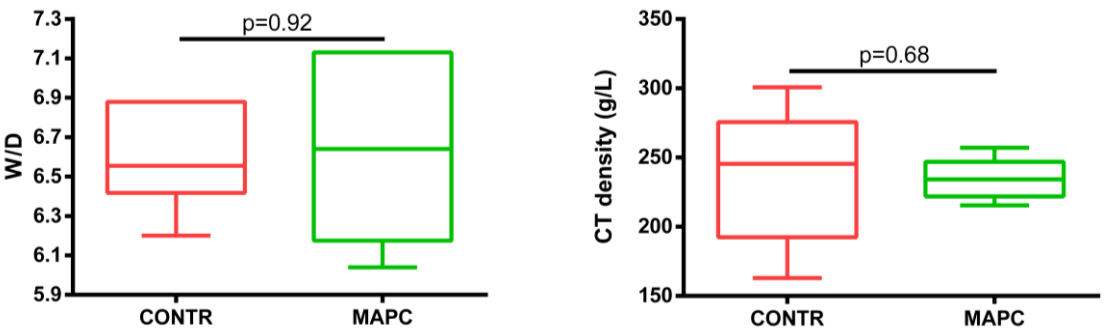

Figure VI.10 – Lung edema, estimated here with W/D and CT density, is not reduced in the MAPC cell group. Data are depicted as boxplot, and compared with a Mann-Whitney test.

Histology

No significant differences were detected in the injury scores for both groups for the presence of congestion (p=0.92), necrotic cells (p=0.70) or influx of neutrophils (p=0.56) (Figure VI.11).

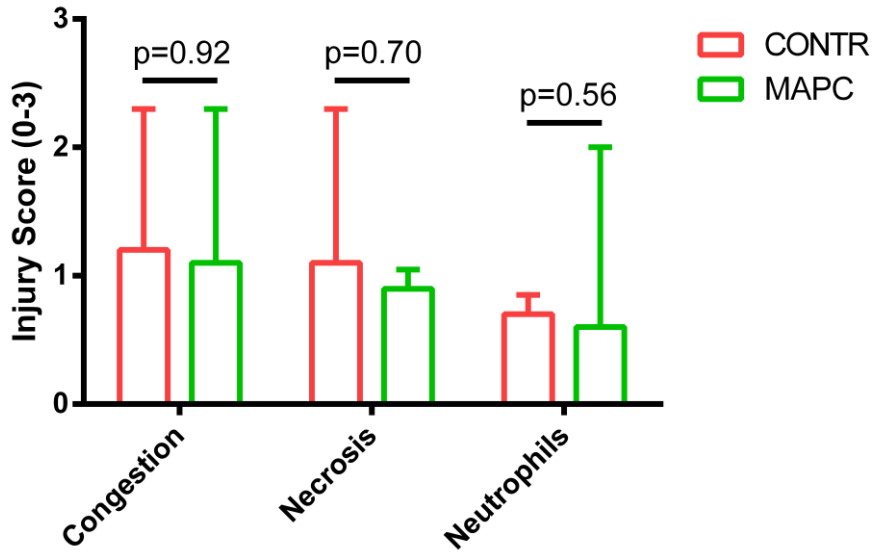

Figure VI.11 – No differences were seen in the injury score for congestion, presence of necrosis and neutrophils.

Inflammation bronchoalveolar lavage fluid

Quantitative determination of IL-1β, IL-4, IL-8, IL-10, IFN-γ, IFN-α and TNF-α in BAL fluid showed a significant reduction of TNF-α, IL-1β and IFN-γ while IFN-α was similar in both groups (Figure VI.12). IL-4, IL-8 and IL-10 were below the detection limit.

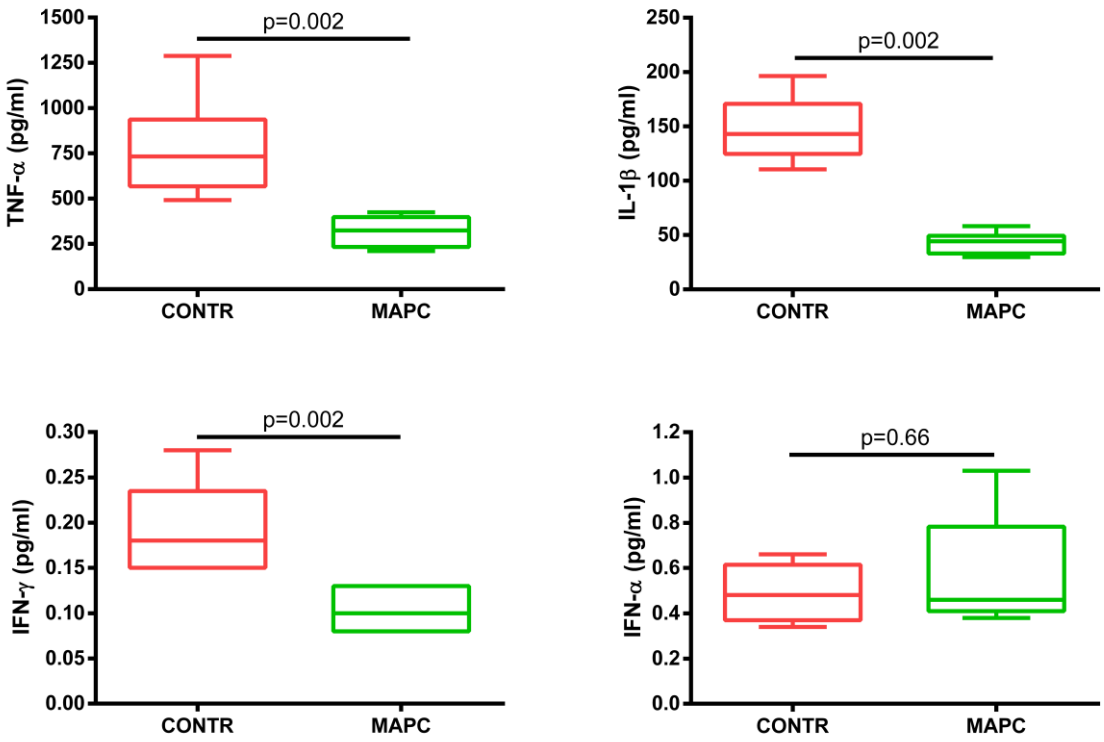

Figure VI.12 – Significant reduction of inflammatory markers TNF-α, IL-1β and IFN-γ in BAL.

Cell pellet analysis of the returned fractions of the BAL fluid showed a trend towards reduction in total cell count in the MAPC cell group (p=0.09). Also, differential cell count showed a significant reduction in neutrophils (p=0.02). Results are depicted as median (25% QI – 75% QI) in Table VI.4.

Table VI.4 – Total cell count (TCC) and differential cell count of BAL fluid cell pellet.

	CONTR	MAPC	p-value
TCC (x10^6 cells/ml)	1.2 (0.9 - 1.5)	0.8 (0.7 - 1.0)	0.09
Macrophages (%)	88.5 (84.2 - 91.9)	95.3 (91.4 - 97.2)	0.06
Neutrophils (%)	7.7 (6.5 - 13.5)	1.7 (1.2 - 4.4)	0.02
Lymphocytes (%)	2.5 (0.8 - 3.9)	3.0 (1.7 - 3.7)	0.79

F) DISCUSSION

In this study, we report the immunoregulatory effects on pulmonary IRI, of MAPC cell administration in the airways. Distribution of 150 x 10^6 MAPC cells in the airways of warm-ischemic porcine donor lungs resulted in a decreased concentration of TNF-α, IL-1β and IFN-γ in the BAL supernatant. Also, a decreased percentage of neutrophils and a trend to a lower total cell count in the BAL cell pellet was observed. The attenuation of this inflammatory response to warm-ischemic injury, was not reflected by an improvement in physiologic parameters, histology or lung edema during EVLP assessment.

Up to now, bone-marrow derived MSC are most frequently used for immunoregulation of the inflammatory response to pulmonary ischemia-reperfusion injury (29,30). MAPC cells are also bone-marrow derived mesenchymal cells and share many similarities with the MSC, however in different culture conditions they adapt different phenotypes (15). The interest in the use of cellular treatment for ischemia-reperfusion injury results from in vitro evidence of their immunoregulatory capacities published in the last decade (31). This immunoregulation is mainly described as the suppression of regulatory T-cells in vitro (16) while the interaction with neutrophils, the most important effector cell in IRI, is largely unexplored. MSC and MAPC share similar immunoregulatory capabilities, however MAPC cells are found to be the most potent (32). Furthermore, MAPC cell therapy is privileged over MSC for clinical translation since their low senescence and high population doubling allows for banking of large batches of cells from a single donor. A clinical grade product of MAPC, MultiStem, is produced by Athersys and is already being tested in Phase I and II clinical trials (Athersys, Cleveland, USA). Therefore, we chose this cell type as bone-marrow derived cellular treatment for attenuation of ischemia-reperfusion injury in lung transplantation. Also, xeno-transplantation and allo-transplantation of these cells, is thought to be safe due to a low expression of MHC-I and lack of MHC-II expression (14,33). These immune-privileged properties avoid recognition by the

recipient's immune system, and therefore, this immune mismatch is generally believed not to be an increased risk for patients.

We used a validated injury model with exposure of the lung graft to 90 minutes of warm ischemia (22). This resulted in an accumulation of lung edema when perfused for 6 hours on EVLP, which is reflected by a high W/D (median 6.6 in CONTR) and high CT-density (median 245 g/L in CONTR). An inflammatory cascade with infiltration of neutrophils was launched upon reperfusion of the donor lung on EVLP, even though we worked with an acellular perfusate. This is shown by an increased percentage of neutrophils in the BAL in the CONTR group (median 7.7% vs 1.7%). Most likely, these cells were trapped in the microvasculature of the lung despite adequate antegrade and retrograde flush of the lung upon procurement. Also, general inflammatory markers such as TNF-α, IFN-γ, IFN-α and IL-1β were increased in CONTR showing activation of the innate immune system with interaction of macrophages, lymphocytes, endothelial cells and epithelial cells.

While Mordant et al could show an IL-8 reduction in the perfusate in their model of MSC therapy during EVLP (13), the detection limit of 0.56 pg/ml for IL-8 was not reached in our BAL sample. Therefore, we cannot speculate on the effect on IL-8 and its neutrophil chemotaxis effect. We did report a reduction in general inflammatory markers involved in the innate immune system, such as TNF-α, IL-1β, IFN-γ and saw a significant reduction in neutrophils in the BAL sample. While these immunoregulatory effects are highly significant, they were not reflected by an improved graft function on EVLP evaluation. The exact effect of this immunoregulatory effect, therefore, has to be further unraveled in a transplant experiment where the lung graft is reperfused with a pulsatile flow of whole blood and the full impact of IRI can be studied.

Also, dose-response studies will have to be further conducted to determine the maximal effect. In this study, an intermediate dose of 3.75 million MAPC / kg body weight was administered.

In mice studies, 0.5 to 2 million cells are usually administered which corresponds to 100 to 200 million cells/kg. These doses are probably irrelevant for clinical practice since we also have to watch out for entrapment of these large cells (15-19µm) in the microvasculature of the lung with micro-thrombi formation, increased PVR and hydrostatic pulmonary edema as potential dangerous consequence. A low dose of 0.24 million cells/kg has been shown not to be effective to tackle IRI as reported in the previous chapter. It might therefore be that one should be looking for an optimal dose instead of a linear dose-response effect.

Finally, we acknowledge that biodistribution of our cells could not be shown. Entrapment and localization of the administered cells will have to be investigated to understand the underlying mechanisms of the immunoregulatory capacities of cellular therapy.

In conclusion, we can state that, although no physiologic effect of immunoregulation was detected during EVLP, we did observe a reduction in pro-inflammatory cytokines and neutrophils in the BAL after MAPC cell distribution in the airways indicating a regulatory effect on the innate immune system. This effect might play an important role in critically modifying the process of PGD early after transplantation (34). The innate immune system is also involved in the etiology of bronchiolitis obliterans syndrome (BOS) (35), and therefore MAPC cell administration early in the process of lung transplantation might have an effect on the development of BOS, the main predictor of long-term outcome. Further experiments will have to elucidate the effect of MAPC cell administration on graft function after transplantation.

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CHAPTER VII
GENERAL DISCUSSION AND FUTURE PERSPECTIVES

A) GENERAL DISCUSSION

Early outcome after lung transplantation is highly dependent on the availability and quality of donor organs. Currently, lung yield remains critically low and organ quality is often limited or unknown when assessed inside the donor chest. Consequently, there is a persistent mortality on the waiting list of 10% and, up to 30% of recipients develop severe primary graft dysfunction after lung transplantation. In order to improve early outcome after lung transplantation, this PhD project focused on novel strategies to increase both number and quality of available donor lungs.

Aggressive donor assessment to increase the donor lung yield

Donor selection nowadays is still based on donor age, chest X-ray, bronchoscopy, gas exchange, ABO compatibility, size matching, smoking history, medical donor history and physical examination of the pulmonary graft at the donor hospital during organ retrieval by an experienced transplant surgeon. With the latter being the most important step to assess for lung injury and transplant suitability (1). Costa et al also suggested to take selective pulmonary vein gases to provide corroborative objective support to the findings at bronchoscopy, palpation and visual assessment since central aortic gases did not always reflect true function of the lungs, having high false-positive rates towards the individual lower lobe gas exchange (2). Current selection criteria are however still largely subjective and have poor predictive power (3). In addition, the selection criteria of the "ideal lung donor" were based on expert opinion and are not based on large prospective, controlled trials. Therefore, all these criteria have to be critically evaluated separately for each donor. Safely liberalizing these strict criteria can only be done in view of the allocated recipient patient. That is, allocating an ECD lung to recipients with a high LAS score could lead to an impaired outcome both on the short- as the long term (4). Nowadays, 60% of lungs transplanted in our transplant program are procured from ECD with excellent outcome. The practice of increasing the donor pool by recruiting also ECD donor lungs, has

been partly stimulated by the introduction of EVLP. EVLP was designed to properly evaluate a donor organ prior to transplantation and allows for transplantation of high-risk donor lungs. Keshavjee et al showed similar early outcome of high-risk donor lungs that were physiologically stable during 4 hours of ex vivo perfusion, compared to recipients transplanted with conventionally selected lungs (5).

However nowadays, EVLP is not a prerequisite to transplant donor lungs from extended-criteria donors. More procurement teams are send out to donor hospitals to critically inspect and reassess the donor lungs inside the recipient chest. Often, these lungs are of excellent quality and don't even need an additional evaluation on EVLP.

The main reason why procurement teams are send out more often, is that traveling distances in Belgium are relatively short compared to other countries. Our transplant center, therefore, has a high organ yield of 38% compared to lower percentages of 10-25% in other transplant centers. In an editorial comment we addressed this issue of assessing more organs inside the donor chest to increase the number of available donor organs (6). Only in a minority of cases, EVLP is used for additional evaluation and preservation of these donor lungs. EVLP might still be able to further increase the donor pool, as several research groups report reconditioning on EVLP of donor lungs that were initially rejected for transplantation (7,8). However, there inclusion criteria for "rejected donor lung" is often based on low PaO_2/FiO_2 only and the potential of EVLP reconditioning might therefore be an overestimation of the true potential. Therefore, we investigated the potential role of EVLP evaluation, preservation and reconditioning and estimated that our current lung acceptance rate of 37.7% could be increased to 44.3% based on a retrospective data base analysis of registered donor data of unused donor lungs.

Donor management to increase lung quality and organ yield

Despite all efforts to increase the number of donor lungs, the difference between organ supply and organ demand is still existent. And with no foreseeable increase in the number of potential

donors, it is therefore of crucial importance that we maximize the organ yield of our current donor pool. Therefore, intensive care physicians are more involved to provide intensive care and organ support to the potential organ donor. Aggressive donor management protocols with pre-set goals have systematically improved organ yield in all solid organ transplant programs (9). Donor management of the DBD donor is based on endocrine support to counteract the collapse of the hypothalamic-pituitary axis, on hemodynamic support with short-acting agents to resuscitate the cardiovascular effects of the autonomic dysregulation following brain death, and on protective ventilatory support with frequent recruitment maneuvers of the lung (10). These strategies affect the pathophysiology of DBD, which is based on a systemic pro-inflammatory response that ultimately can lead to organ dysfunction (11). Brain death leads to massive release of catecholamines and pro-inflammatory mediators in an attempt to overcome hypoperfusion of the brain. However, this response has a catastrophic systemic effect with failure of the microcirculation, leukocyte recruitment, release of ROS, etc (12).

Donation after circulatory death (DCD) describes the retrieval of organs for the purposes of transplantation that follows death confirmed using circulatory criteria, rather than brain death criteria. Consequently, organ injury in donation after circulatory death is based on the warm-ischemic time (WIT) rather than on the pathophysiological consequences of brain death. WIT is the time when there is insufficient circulation to the organs to deliver a minimum amount of oxygen and nutrients (13). The incidence of graft complications after transplantation is related to the length of this WIT and tolerance to WIT is organ-specific. Lungs could tolerate up to 60 minutes of warm ischemia (14,15). Ischemia-reperfusion injury is inherent to the process of organ transplantation and also additional cold ischemia further contributes to the development of PGD in DCD. The pathophysiology of organ injury in DBD and DCD is therefore not completely similar (16). The question that follows is whether current organ support measurement in DBD donors also apply to organ support for DCD donors. Unfortunately, there

is a paucity of randomized clinical trials since organ donor management has not been a top priority in the ICU practice up to now (17), and evidence is mostly provided by observational studies only. Generally, it is agreed upon that aggressive donor DBD management protocols do make a difference in the number of organs transplanted. However, whether these findings will also apply on DCDs remains an open question due to the different pathophysiology of lung injury and the different ethical context. The dead donor rule (18) must be respected at all times, so no treatment can be administered with the aim on organ quality improvement as long as the donor does not meet valid criteria for death. It is only permissible after death declaration, either by the criteria of brain death or by permanent cessation of circulatory function. Criteria for determination of death should be followed meticulously for each individual donor, and no rules should be bend to increase the success rates of procuring good quality organs. Even though an altruistic viewpoint might seem tempting to defend the organ optimization in a DCD donor prior to death, since the consent for organ donation has been agreed upon, and the patient suffers from an end-stage pathology with natural death that will occur soon otherwise. The critical question should probably be to consider some treatments that do not hasten death, which is the reason why some countries already allow heparin administration. A retrospective study of the ISHLT DCD study group also revealed that 93% of centers do administer steroids before the withdrawal of life support therapies. This practice might be based on small observational studies in DBD donation that report improved donor hemodynamics, oxygenation, increased organ yield and even improved recipient and graft survival (19). Also, the steroids might have been administered for other reasons or might have been a part of general ICU practice (eg neuroprotection). However, data on the effect of steroids in DCD organ donation was lacking. With our porcine DCD model, we are the first to provide beneficial evidence to support the practice of steroid administration prior to withdrawal of life support therapies in donation after circulatory death.

Assessment and preservation on EVLP

Worldwide, clinical experience with EVLP is growing since the beginning of the 21st century and different clinical protocols are validated. The largest clinical experience comes from the Toronto group, which has the largest clinical EVLP cohort of over 200 EVLP cases of both SCD and ECD (5,20). Also the LUND protocol is actively used. Henriksen et al recently published the Danish experience with improved PaO_2/FiO_2 after EVLP in 8 cases (21). The NOVEL trial (multi-center) in the United States (US) works with the XVIVO technology and is still recruiting unusable donor lungs for ex-vivo reconditioning to test clinical effectiveness and improved survival, to increase lung transplant activity in the US. Two large multicenter randomized controlled trials are being performed with the portable EVLP technology of the OCS™ Lung. The Inspire Trial, designed as a safety study, included SCD lungs and met all of its non-inferiority end-points including a significant reduction of PGD grade 3 (22,23). The Expand trial includes ECD lungs and is still ongoing. EVLP technology requires many resources, both technical and human. The expertise of a well-trained EVLP physician or perfusionist has to be available at all times for the procedure itself and to evaluate the graft quality on EVLP. It might therefore be advisable to combine these resources and expertise in EVLP centers. Currently, the Perfusix trial is assessing the efficiency of such an EVLP center in the US and Canada (24). Analysis of the recently completed and ongoing clinical EVLP trials with both SCD and ECD lungs will have to clarify which lungs and patients could benefit most of this EVLP technology so that resources can be applied in a cost- and clinical-effective manner. Also in unique, high-risk cases, EVLP can be applied to safely prolong the "out-of-body time" of donor lungs as we have shown in our case report of a combined liver-lung transplantation with normothermic preservation of the lungs on EVLP. To implement EVLP in routine lung transplant preservation strategies, a risk score analysis could be performed incorporating the main risk factors for PGD development (25): 1) donor age, smoking 2) Single-

lung transplant procedure, cardiopulmonary bypass expected during procedure 3) recipient diagnosed with pulmonary hypertension, sarcoidosis, obesity. These risk factors could all be individually weighed and combined to a risk score for PGD development. In this way, we can avoid to put all donor lungs on EVLP and apply EVLP only to those cases that could benefit most from it. In many centers, lung yield could already be increased by sending more retrieval teams to the donor hospital, even without EVLP. Dedicated retrieval teams and transportation costs should also be taken into account, but are still more profitable compared to EVLP technology.

Three EVLP protocols are available (see Table I.4 Introduction, p.17) for clinical EVLP. These techniques differ in portability, pump type, ventilator settings, pump flow, perfusate composition and pressure control of the left atrium (open vs closed). Despite these differences, all are based on the principle of physiological evaluation, protective lung ventilation and high-oncotic perfusate composition. No studies are available comparing these 3 techniques to one another. Data on individual aspects of these protocols are available but, they do not allow complete comparison. For example, Linacre et al report a superior graft function when there is a closed atrium with controlled left atrial pressure compared to open drainage of the left atrium (26). However, groups with elaborate experience with an open left atrium on EVLP, also report excellent results after lung transplantation (23). Since prolonged cold ischemia contributes to ischemia-reperfusion injury, it is found to be protective to avoid cold ischemia with a portable normothermic machine perfusion device such as the OCS^{TM} Lung. However, other groups report better results if a short period of cold ischemia is introduced prior to normothermic evaluation (27). After a 3-year experience with both the Toronto, Lund and OCS^{TM} protocol, it seems that the success of EVLP preservation and reconditioning is could be considered independent of the used technique or protocol, but related to the experience and trust build up on one technique. Research groups should therefore not only focus their resources in comparing

these protocols, but should extend their experience in trained organ perfusion teams and refinement of their own technique.

Follow-up on the trend of physiological parameters is still the mainstay of evaluation during EVLP in all available protocols. More particular, we look at the pulmonary vascular resistance, oxygenation and dynamic lung compliance and want to see an improvement or steady state. However, which parameters are most predictive for organ function after transplantation, is still under debate. Some groups prefer to base their evaluation on oxygenation and lung compliance (28), other include pulmonary vascular resistance and oxygenation (22). In all protocols, lung compliance seems the first parameter to indicate lung injury, also in our experimental studies (29–31). It is known that in acellular perfusion protocols, oxygenation is not the most reliable parameter to evaluate lung injury in an ex-vivo setting (32). In an acellular perfusate, the shunt-induced reduction in PO_2 at the outflow due to lung edema is less pronounced since there is a linear relationship between oxygen content and PO_2. Whereas in cellular, Hb-containing perfusate there is a sigmoidal relationship between oxygen content and PO_2 and therefore shunt-induced reduction in PO_2 will be more pronounced and more easily detected (32). Which type of perfusate (cellular or acellular) we should use, is still a subject of debate. Only pre-clinical studies have been published so far comparing the use of an acellular or cellular perfusate. Two studies report similar physiological parameters (PVR, compliance, oxygenation) and amount of lung edema (W/D) after prolonged periods of lung preservation with either an acellular or a cellular perfusate (addition of packed RBCs). Data on whole-blood perfusion models have not been published yet but might be superior due to the beneficial effect of plasma on endothelial function as has been published in heart perfusion models (33). Despite the buffering and oxygen delivery advantages, the use of blood as part of the perfusate is more infrequent in reporting centers (34). Differences in shear stress and prolonged exposure time of the blood to the tubing and pump device can lead to hemolysis of the RBCs in the blood based perfusate. Therefore,

we work with an acellular perfusate for our prolonged preservation and reconditioning experiments. The perfusate used in our experiments, is a laboratory tested low-potassium, high-oncotic albumin-containing acellular perfusate that is similar to Steen Solution (XVIVO).

Also secretion of biochemical markers such as endogenous nitric oxide synthase levels (35), endotheline-1 (36), IL-1beta (37) and IL-8 (38) during EVLP may predict acceptable allograft function. That is, lungs perfused on ex-vivo lung perfusion have a specific cytokine expression profile that can be used in future analysis to predict outcome after lung transplantation (39). These parameters may become more important in the future to complement the physiologic evaluation EVLP.

W/D measurements are still the golden standard for estimation of lung edema. In addition, we introduced CT-density measures that give an average density of the total lung instead of a W/D measurement of only a small piece of the lung (40). In all our experiments, this CT-density measurement correlated nicely with W/D. For these calculations, lungs were frozen solid and CT-scanned afterwards however, it is also feasible to scan the lung after the experiment to incorporate the density measurement as an additional parameter in your final evaluation of transplantability. We believe that performing high-tech imaging could improve the quality of our ex-vivo evaluation of donor lungs. But even more strongly, it might be easier to perform a CT-scan of the donor to assess donor lung transplantability prior to EVLP. This will avoid unnecessary transport costs of the retrieval team to retrieve eg emphysematous or severely infected donor lungs. On the other hand, there is of course the risk that we will reject donor lungs that we would have otherwise transplanted if there was no CT-scan available. CT-imaging will give us more information on the donor lung, however, for active donor reconditioning and evaluation of the treatment effect, we will still need EVLP in the near future. A pilot study was already conducted in our laboratory (Figure IV.4, chapter IV.A), where a CT-scan was conducted as an additional evaluation parameter prior and after EVLP to evaluate the possible

improvement in lung quality. Future research will elucidate the value of CT-imaging in assessing donor organ suitability with or without EVLP.

Prolonged ex-vivo lung perfusion model with reproducible lung injury

In order to evaluate the effect of a reconditioning therapy, we have to maximize the time period where the graft is exposed to this therapy and have to incorporate an observation period that is long enough to detect a difference in the development of sufficient ischemia-reperfusion injury resulting in primary graft dysfunction. In our laboratory, EVLP used to be employed for only a maximum of 2 hours to evaluate physiological parameters (16,41–43). However, a peak of inflammation is not reached up to 3 hours of perfusion which correlates with the absence of BAL neutrophilia in experimental perfusion protocols (44,45). Therefore, for the first time in our laboratory setting, we prolonged the evaluation period on EVLP to six hours in our experimental set-up. Extending the ex-vivo lung perfusion time safely without inflicting injury to the donor lung requires correct pressure monitoring at the in- and outflow (left atrium) (26), protective lung ventilation, a high-oncotic perfusion solution and a gradual rewarming plus perfusion of the graft (46). Additionally, to study ischemia-reperfusion injury a reproducible injury model had to be validated. Lung injury in DCD organ donation is based on WIT and since our laboratory had most experience in this field, we continued with these warm ischemic injury models (42,47,48). An initial WIT of 120 minutes, seemed to be too severe for porcine donor lungs which was reflected by an excessive amount of lung edema and the presence of necrosis on pathology (Chapter V.A). Therefore, the following experimental study designs incorporated a WIT of 90 minutes (Chapter III, VI.A, VI.B) (37). In addition, we also validated a prolonged cold ischemic injury model in Chapter V.B that is also used by other research groups (49,50). The development of a stable and reproducible injury model is difficult to standardize in animal models. There is a lot of variation in time between reported studies and even between investigators. Although it might seem simple to perfuse and ventilate a donor

lung, detailed care has to be provided to the donor lung on EVLP. Meticulous pressure, volume, flow and temperature monitoring are pivotal in order to successfully preserve donor lungs on EVLP without inflicting injury that would result in edema formation.

Organ reconditioning on EVLP

Organ reconditioning, the improvement of organ quality, is the main topic in EVLP literature nowadays. Both simple (such as antibiotics, steroids) and more advanced (such as stem cells, viral vectors) treatment options have been proposed as ideal reconditioning strategies on EVLP. Within this PhD project, several strategies have been put forward to increase the number and quality of donor lungs. First of all, physical examination of the pulmonary graft at the donor hospital during organ retrieval by an experienced transplant surgeon allows for a more grounded decision to put lungs on EVLP. Additional evaluation could lead to acceptance of the pulmonary graft for transplantation. Secondly, also perfusion and ventilation itself on EVLP with reduction of neurogenic lung edema, reduction of the microbial load and recruitment of atelectatic areas could lead to acceptance of the pulmonary graft. Thirdly, we have investigated if active treatment with anti-apoptotic and anti-inflammatory agents on EVLP could lead to an improved quality of the donor lungs (= "reconditioning"). Noble gases Ar and Xe could be of particular interest for active organ reconditioning based on their organ protective effects seen in other research fields (51–53). However, further research in DBD transplant models has to be conducted to validate such an organoprotective effect in lung transplantation. Cellular treatment with MAPC in the airways of warm-ischemic porcine lungs was able to regulate the innate immune response however failed to induce an improved lung physiology. Further research will have to be performed to elucidate the full effect of this immunoregulatory effect after lung transplantation with an optimal dose of MAPC cells administered in the airways.

Several experimental pre-clinical studies show beneficial effects of other individual treatment options, and even some clinical case reports have been published with improved organ quality

and good patient outcome. However, these strategies have not found their way into standard operating procedures for EVLP preservation and reconditioning prior to transplantation. In concordance with clinical pathology, different types of injury require different treatment options. Therefore, a decision tree that incorporates donor medical history, acquired lung injury, PGD risk of the recipient etc. could help to apply the correct treatment for its specific target. For example, lung emboli cannot be treated with antibiotic treatment or stem cells, but can be dissolved with a fibrinolytic agent such as urokinase (54). However, inflammation and infection following aspiration could be treated with surfactant (55) or stem cells (56) and ischemic injury with viral vector or gene therapy (57). One therapy that fits all is therefore not feasible, but it might be that there is one therapy for each injury type separately. Research groups worldwide should combine their experiences to design an organ-tailored treatment algorithm for ex-vivo organ reconditioning prior to transplantation.

Is there need for further research with noble gases for organ protection?

Several authors have already summarized the individual neuroprotective results for Ar (58). In a meta-analysis, De Deken et al showed that Xe had the most consistent effects, being neuroprotective in rodents, cardioprotective in rodents and pigs, and renoprotective in rodents (51).

Often, an organ protective effect of noble gases is studied in combination with hypothermia. Broad et al showed neuroprotection by a combination of Ar and hypothermia, in a piglet model of neonatal asphyxia (59) whereas Zhao et al showed that Argon mediates neuroprotection in combination with hypothermia in a hypoxic-ischemic injury model in rats (60). Also for Xe, combinational therapy with hypothermia has been shown to be organoprotective in different injury models (61–64). It might therefore be that most beneficial organoprotective effects of noble gases can be achieved by an enhancement of the protective effect of hypothermia on hypoxic or ischemic tissue. Niemann et al showed that hypothermia of the organ donor alone,

also gives protection against ischemia-reperfusion injury and can increase the organ yield (65). Therefore, the combination of hypothermia and noble gases will have to be investigated further in solid organ transplantation. Introducing hypothermia plus noble gas ventilation is, however, cumbersome. And it might be more time- and cost-efficient to investigate simpler treatments for donor organ optimization.

In contrast, also protective effects of noble gases have been shown in normothermic conditions already (66–69). Therefore, the organ protective effect of noble gases cannot be contributed to an enhancement of the protective effect of cooling alone. Unfortunately, most data come from in vitro and small-animal models and will have to be confirmed in larger animals such as pigs. Also, timing, dosing and duration of noble gas ventilation has to be further explored.

In our results, the absence of a protective effect on pulmonary ischemia-reperfusion effect should be embedded in this broader discussion: whether the organoprotective effects of noble gases might be due to an enhancement of hypothermic protection or whether the chosen cold-ischemic injury model was not appropriate to study organ protection by noble gases. That is, injurious processes involved in brain damage that could harm donor organs, such as apoptosis, excitotoxicity, brain-dead induced inflammation and hemodynamic instability might be subjectable for attenuation by noble gases by modulation of the MAPK-ERK1/2 pathways. Attenuation of IRI in a brain-dead model should therefore be investigated. In addition, to fully study the effect of our noble gas treatment on IRI we should transplant reconditioned lungs to have recruitment of neutrophils upon reperfusion in the recipient, the most important effector cell in the injurious process of IRI. Our reperfusion model with whole blood can only be seen as a surrogate for transplantation since we merely mimicked transplantation, and the full effect on primary graft dysfunction will have to be explored in an allotransplant model with recruitment of many more neutrophils upon reperfusion.

Immunoregulatory mechanisms of mesenchymal cell treatment on lung injury

In the beginning of MAPC research, most studies referred to the differentiation of the MAPC into specific cell types for tissue repair. However, engraftment and differentiation of these cells is not found to be the major effector function of this cellular therapy since Gupta et al showed that after 48 hrs there was less than 5% engraftment of labeled MSCs in injured mice lungs (70). Much of the current interest in mesenchymal cell products comes from the ability to home to injured tissue and secrete paracrine factors such as growth factors, factors regulating epithelial and endothelial function, and anti-inflammatory cytokines. The administration of mesenchymal cells leads to a reduction of inflammatory cell recruitment (70,71). The pathophysiology of organ injury following ischemia-reperfusion injury is based on an inflammatory cascade with massive activation of macrophages, infiltration of neutrophils and nourishment of a pro-inflammatory cytokine profile. The therapeutic effect, of mesenchymal cells is most likely not primarily derived from a pure and selective anti-inflammatory effect but from a regulatory effect on the immune response following transplantation. Several candidate mediators have been identified so far, first: attenuated lung injury can be partially mediated by inhibition of TLR signaling, a key player in activation of the innate immune system (72). Also Wang et al recently showed that interaction of bone-marrow derived mesenchymal cells with TLR signaling is a key player in modulating acute lung injury (73). In this way, activation of macrophages in the early inflammatory IRI response, through MAPK and NF-kB signaling pathways, is inhibited and the devastating cascade of inflammatory lung injury is attenuated (74). This effect is most likely not cell-contact dependent, but depending on release of prostaglandins such as PGE_2 that acts on macrophage-receptors (70,75). Second, mesenchymal stem cells are known to produce epithelial specific growth factors such as KGF. KGF can restore alveolar fluid clearance in rodent models through activation of the PI3K/AKT/mTOR signaling pathway (76). AKT phosphorylation can result in decreased apoptosis of monocytes

(77), which can lead to higher bacterial clearance and a therapeutic effect in case of infection (78). Third, clearance of developed lung edema can be inhibited by a high concentration of inflammatory cytokines that block fluid clearance in the lung (79). A reduction in several pro-inflammatory cytokines such as TNF-α, IL-1β, IFN-γ as seen in our experiments could therefore lead to an improved alveolar fluid clearance. Although we did not investigate alveolar fluid clearance in our study, we did not detect a difference in its end-results: lung edema was not reduced in injured lungs treated with MAPC. And while Mordant et al indicate that reduction of circulating IL-8 levels is the main factor for reducing organ damage in an ex-vivo lung perfusion model (49), other groups, including our own, did not found this in their results (80). So although some involved pathways have been identified, there is paucity of pre-clinical models with convincing results and more research in this field will have to elucidate the true effect of mesenchymal cells on ischemia-reperfusion injury and the inflammatory environment of the donor lung.

As mentioned, we also showed a reduction of cytokines in the bronchoalveolar lavage fluid in Chapter VI.B. Unfortunately, this was not reflected by an alteration in the composition of the cells present in the BAL fluid. Nevertheless, reduced levels of pro-inflammatory levels such as TNF-α and IFN-γ do support the inhibition of macrophage activation. However, the effects of decreased macrophage activation on ischemia-reperfusion injury could not be studied to its full extent since lungs were only perfused with an acellular perfusate in our ex-vivo perfusion model. Future studies should focus on the potential physiological effect of mesenchymal cell product treatment in pre-clinical transplant models.

This thesis project was focused on the early inflammatory phase after reperfusion of the donor organ. Other clinical indications in solid organ transplantation could be the prevention of acute transplant rejection and improvement of long-term survival with a minimum of immunosuppression to decrease the incidence of chronic rejection. These effects are not based

on the innate immune effects described earlier, but on the adaptive immune system. Preliminary results of a MSC treatment for chronic lung allograft dysfunction study showed a doubling of T-regulatory cells and a significant increase in epidermal growth factor (EGF) in patients receiving low-dose MSC (81). Pro-inflammatory Th1-cytokines and chemokines were significantly decreased. Zhao et al also showed that mesenchymal stem cells could mitigate the development of chronic lung allograft dysfunction by preventing luminal obliteration after transplantation. This was obtained by protection against epithelial cell loss through promoting epithelial progenitor cells and modulation of the immune reaction by inducing regulatory T-cells and suppression of cytotoxic effector T-cells (82). The effect of mesenchymal cells on effector and regulatory T-cells is also why these cells have gained interest to treat and prevent graft-versus-host-disease (83) and allograft rejection in solid organ transplantation (84). The implications of a reduction in inflammatory cytokines observed in our study (Chapter VI.B), could therefore also have implications on the long-term survival and prevention of chronic lung allograft dysfunction.

B) CONCLUSION AND FUTURE PERSPECTIVES

We have shown that an increased number and quality of donor lungs can be obtained by introducing simple measures to optimize the donor phase. By sending out more retrieval teams to the donor hospital, more organs will be evaluated inside the donor chest for their transplantability rather than evaluating their suitability on registered donor data only (Chapter IIIA). In this way, more donor organs will be procured even without additional evaluation on ex-vivo lung perfusion (EVLP). EVLP does come in handy when evaluating an interventional effect to improve organ quality. We have shown that pre-arrest corticosteroid administration to a DCD donor (donation after circulatory death) that is continued during EVLP, results in improved pulmonary graft function. We are the first group to report comparative experimental data to support the practice of pre-arrest steroid administration in DCD donation programs (Chapter IIIB).

With a retrospective donor database analysis, we validated that ex-vivo lung perfusion (EVLP) indeed holds great potential to increase the number of transplantable donor lungs by including carefully evaluated and optimized extended-criteria donor lungs in the donor pool (Chapter IV.A). Our study resulted in improved donor data registration to further improve our results and will provide us with insights in the causes of donor lung decline in the future. Hereby, we want to stimulate our transplant physicians, transplant coordinators, transplant surgeons and anesthesiologists to invest in extended-criteria donor lungs by carefully evaluating and even selecting them as candidates for EVLP.

We also showed that ex-vivo lung perfusion is widely applicable in for example combined solid organ transplantation or pediatric transplantation. We highlighted that anesthesiologists are often confronted with the remote impact of ischemia-reperfusion injury which results in organ failure. Their focus should therefore be extended from managing the peri-operative challenges

to an interest and investment in strategies to reduce ischemia-reperfusion injury by optimizing organ preservation (Chapter IV.B).

Ex-vivo lung perfusion holds great potential to increase both number and quality of donor lungs and we demonstrated that this technology can be used as a platform for reconditioning. We investigated two innovative treatment strategies to attenuate the effects of ischemia-reperfusion injury on pulmonary graft function. Noble gases Ar and Xe, and bone-marrow derived multipotent adult progenitor cells (MAPC) were selected for their pro-survival and immunoregulatory properties.

We showed that ex-vivo reconditioning with Ar and Xe post-injury (postconditioning) was not successful in a porcine model of warm-ischemic pulmonary injury (Chapter V.A). Also prolonged exposure to Ar prior, during and after cold ischemic injury could not alleviate ischemia-reperfusion injury (Chapter V.B). Organoprotective effects of noble gases in other injury models of brain and myocardial injury were mostly described in combination with hypothermia. Therefore, the amplifying effect of noble gases on the hypothermic protective effect against ischemia-reperfusion injury should be further investigated together with the pathophysiological working mechanisms.

We identified that intratracheal administration of MAPC resulted in less edema formation in a porcine warm ischemic injury model. However, no significant improvement in lung physiology or inflammation could be detected (Chapter VI.A). In a second work package, higher dosing of MAPC in the airways resulted in a significant reduction of neutrophils and inflammatory cytokines in bronchoalveolar lavage fluid. This attenuation of the inflammatory cascade of ischemia-reperfusion injury, however, was not reflected in improved lung physiology (Chapter VI.B). The impact of the immunoregulatory effect of MAPC on the clinical entity of primary graft dysfunction early after lung transplantation should be further unraveled together with the pathophysiological working mechanisms and biodistribution of these cells.

In conclusion, we identified that EVLP is a valuable strategy to increase the number and quality of donor lungs for transplantation. Noble gases failed to improve lung graft function in normothermic conditions even after prolonged exposure. However, MAPC distributed in the airways in a high dose (3.75 million/kg) are able to alter the inflammatory environment of the lung. Even though they failed to induce an improved lung physiology, the impact and pathophysiology of this immunoregulatory effect should be unraveled to improve organ quality. We showed that pre-arrest donor management with corticosteroids resulted in improved graft function. Therefore, donor management strategies should be highlighted in transplant programs to improve outcome after lung transplantation. Also, transplant teams should be encouraged to invest in identifying, carefully evaluating and procuring as many qualified donor lungs as possible to increase the lung yield and our transplant activity.

In this way, we can improve organ quality, further decrease the persisting organ shortage and ameliorate early outcome after lung transplantation.

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SUMMARY

Lung transplantation is a lifesaving treatment option in well-selected patients with end-stage lung disease. Due to the successes of lung transplantation, transplant programs are expanding rapidly worldwide. However, two major problems impede optimal outcome early after lung transplantation. First, 10 to 30% of all lung recipients develop primary graft dysfunction, which is the leading cause of early morbidity and mortality after lung transplantation. PGD is a form of organ failure, and is the end-result of an injurious process that already starts in the donor, continues during preservation of the donor organ and peaks upon reperfusion in the recipient. This results in accumulation of lung water and impaired oxygen transport. Secondly, many donors do not match the strict criteria of lung donation. Therefore, there are more patients listed for lung transplantation than there are donor lungs available, and consequently 12% of listed patients die while waiting in Europe. In addition, those lungs that are available are often of limited quality which again leads to a higher incidence of graft failure and limited early outcome.

This PhD project focusses on two separate time intervals prior to the lung transplant procedure that influence the number and quality of donor lungs. During the donor phase, a suitable donor organ is selected after careful evaluation by the transplant team to obtain optimal organ quality. Secondly, preservation of the donor organ largely determines the organ quality.

In this project, we commented that more donor organs can be made available for lung transplantation when more retrieval teams are send out to inspect the organ quality inside the donor rather than making a selection on medical donor information only. Also, we are the first to provide evidence to support the current clinical practice of administrating corticosteroids to donors who die of circulatory arrest, since this results in improved organ function after lung transplantation.

To evaluate graft function prior to transplantation, ex-vivo lung perfusion (EVLP) was developed. With this technique, a preservation solution is pumped through the lung while it is

ventilated so we can evaluate the organ physiologically outside the body. It also offers the opportunity to treat the donor organ and evaluate the effect or the treatment prior to transplantation.

By analyzing data of all donor organs that were not used for transplantation, we could show that there is a large potential for EVLP to make more donor lungs available for transplantation by carefully evaluating them and improve them prior to transplantation. We also described our experience in a pediatric combined liver-lung transplantation where the lungs were kept on EVLP before they were transplanted. We showed that EVLP is also possible in unique cases such as this one and stimulated anesthesiologists to be more involved in research of optimal preservation methods of donor organs to improve their quality.

We investigated the effect of administrating noble gases and multipotent adult progenitor cells (MAPC, bone-marrow derived stem cells) on our EVLP device to improve lung quality. While we could not detect a beneficial effect of noble gases on the lung, we did show that the inflammatory process that follows lung injury was less pronounced when MAPC cells were administered in the airways of donor lungs. The impact of this effect will have to be clarified in future experiments.

SAMENVATTING

Longtransplantatie is de finale behandelingsoptie voor patiënten met ernstig longlijden. Omwille van het succes van longtransplantatie breiden longtransplantatie programma's wereldwijd snel uit. Spijtig genoeg zijn er twee grote problemen die optimale resultaten vroeg na longtransplantatie belemmeren. Ten eerste ontwikkelen 10 tot 30 procent van alle longtransplantpatiënten primaire greffe dysfunctie (PGD), wat de voornaamste oorzaak van vroege morbiditeit en mortaliteit is na longtransplantatie. PGD is een vorm van orgaan falen, en is het eindresultaat van een schadeproces dat reeds start in de donor, verdergaat tijdens de bewaring van het donororgaan en zijn hoogtepunt bereikt tijdens de doorbloeding van het donororgaan in de ontvanger. Dit resulteert in de opstapeling van longwater en een belemmerd zuurstoftransport. Ten tweede voldoen vele orgaandonoren niet aan de strikte criteria van longdonatie. Hierdoor staan er meer patiënten op de wachtlijst dan dat er donorlongen beschikbaar zijn, en sterven er zelfs 12% van de patiënten op de wachtlijst in Europa. Daarenboven zijn de organen die wel beschikbaar zijn vaak van onvoldoende kwaliteit wat opnieuw leidt tot een hogere incidentie van orgaanfalen en verminderde outcome in de eerste dagen na longtransplantatie.

Dit PhD project focust op twee tijdsintervallen vóór de longtransplantatie procedure die het aantal en de kwaliteit van de longen kan beïnvloeden. Tijdens de donorfase wordt een geschikt donororgaan geselecteerd na grondige evaluatie door het transplantteam om optimale kwaliteit te kunnen bekomen. Tijdens de bewaringsfase van het orgaan, bepalen de condities waarin de longen bewaard worden sterk de kwaliteit van het orgaan.

In dit project werd er becommentarieerd dat er meer donororganen beschikbaar gesteld kunnen worden door meer teams uit te sturen om de longen te onderzoeken in de donor zelf in plaats van donororgaan selectie louter te laten afhangen van medische donorgegevens. Ook leverden we als eerste evidentie aan, om de huidige praktijk van corticosteroïden behandeling bij donoren

die sterven aan circulatie stilstand, te onderlijnen, gezien dit resulteert in een betere werking van de donor longen.

Om de functie van het te transplanteren orgaan te evalueren vóór het getransplanteerd wordt, werd de techniek van ex-vivo longperfusie (EVLP) ontwikkeld. Bij deze techniek wordt er een preservatievloeistof door de donorlongen gepompt en worden ze geventileerd zodat ze fysiologisch geëvalueerd kunnen worden. Dit biedt ook de mogelijkheid om donororganen te behandelen buiten het lichaam en het effect van de behandeling te evalueren voor ze geïmplanteerd worden in de patiënt.

Door een analyse te maken van alle donororganen die niet gebruikt werden voor longtransplantatie, zagen we dat er een groot potentieel is voor EVLP om meer longen beschikbaar te stellen voor transplantatie door ze aanvullend zorgvuldig te evalueren en te verbeteren voor transplantatie. We publiceerden ook onze ervaring met een pediatrische gecombineerde lever-longtransplantatie procedure waarbij de longen op EVLP bewaard werden voor ze getransplanteerd werden. We toonden hierbij aan dat EVLP ook mogelijk is voor uitdagende casussen zoals deze en stimuleerden anesthesiologen om meer betrokken te zijn in onderzoek naar de optimale bewaarmethodes van donororganen om te kwaliteit te verbeteren.

We onderzochten het effect van toedienen van edelgassen en multipotent adult progenitor cells (MAPC, beenmerg stamcellen) op onze EVLP-machine om de kwaliteit van de donorlongen te verbeteren. We hebben geen gunstig effect van edelgassen kunnen aantonen, maar toonden wel aan dat de ontstekingsreactie die volgt op orgaanschade, minder uitgesproken was indien MAPC-cellen werden toegediend in de luchtwegen van donorlongen. De impact van dit effect zal verder uitgeklaard moeten worden in de toekomst.

CURRICULUM VITAE

An Martens was born on March 26th, 1988 in Herentals, Belgium. After getting her bachelor degree in Medicine at the University of Hasselt (Belgium) in 2009, she graduated magna cum laude from Medical School at the Katholieke Universiteit Leuven (Belgium) in 2013. She started a research fellowship in the Laboratory of Anesthesiology and Algology, embedded in the Lung Transplant Unit, at the Katholieke Universiteit Leuven, in preparation of her doctoral thesis under promotorship of Prof. Dr. Arne Neyrinck, Prof. Dr. Dirk Van Raemdonck and Prof. Bart Vanaudenaerde. Her research focused on improving both number and quality of donor lungs by stimulating transplant teams, donor management, ex-vivo lung perfusion and the application of noble gases and bone-marrow derived cellular treatment. Her scientific work resulted in several awards, abstracts and peer-reviewed publications.

She will resume her residency in Anesthesiology at the University Hospitals Leuven, under supervision of Prof. Dr. Marc Van de Velde in February 2017.

LIST OF PUBLICATIONS

A) Publications in internationally reviewed scientific journals

Martens A, Boada M, Vanaudenaerde BM, Verleden SE, Vos R, Verleden GM, et al. Steroids can reduce warm ischemic reperfusion injury in a porcine donation after circulatory death model with ex vivo lung perfusion evaluation. Transpl Int. 2016 Nov;29(11):1237–46.
(DOI: 10.1111/tri.12823)

Martens A, Neyrinck A, Van Raemdonck D. Accepting donor lungs for transplant: let Lisa and Bob finish the job! Eur J Cardio-Thoracic Surg. 2016 Nov;50(5):832–3.
(DOI: 10.1093/ejcts/ezw261).

Martens A, Montoli M, Faggi G, Katz I, Pype J, Vanaudenaerde BM, Van Raemdonck DE, Neyrinck AP. Argon and xenon ventilation during prolonged ex vivo lung perfusion. J Surg Res. 2016 Mar;201(1):44-52
(DOI: 10.1016/j.jss.2015.10.007)

Martens A, Ordies S, Vanaudenaerde BM, Verleden SE, Vos R, Verleden GE, Verbeken E, Van Raemdonck DE, Claes S, Schols D, Chalopin M, Katz I, Farjot G, Neyrinck AP. A porcine ex vivo lung perfusion model with maximal argon exposure to attenuate pulmonary ischemia-reperfusion injury. Accepted for publication in Medical Gas Research; Jan 2017

Submitted for publication:

Martens A, Van Raemdonck D, Smits J, Verleden SE, Vos R, Vanaudenaerde BM, Verleden GM, Degezelle K, Desschans B, Neyrinck AP. A retrospective database analysis to evaluate the potential of EVLP to recruit declined lung donors. Submitted for publication

Martens A, Pirenne J, Van Raemdonck D, Vanaudenaerde B, Vos R, Verleden GM, Verleden SE, Neyrinck AP. Ex-vivo lung preservation in pediatric combined liver-lung transplantation: a case report. Submitted for publication

Martens A, Ordies S, Vanaudenaerde BM, Verleden SE, Vos R, Van Raemdonck D, Verleden GE, Roobrouck V, Claes S, Schols D, Verbeken EK, Neyrinck AP. Immunoregulatory effects of multipotent adult progenitor cells in a porcine ex-vivo lung perfusion model. Submitted for publication

B) Abstracts presented at international conferences

A. Martens, B.M. Vanaudenaerde, S.E. Verleden, R. Vos, D.E. Van Raemdonck, G.M. Verleden, C. Verfaillie, A.P. Neyrinck. Multipotent adult progenitor stem cell administration in a porcine model of ex vivo lung perfusion. Transpl Int 2015 Nov;28:224.
Presented at the 17th ESOT Congress, September 2015, Brussels (Belgium)

A. Martens, D.E. Van Raemdonck, B.M. Vanaudenaerde, S.E. Verleden, G.M. Verleden, R. Vos, A.P. Neyrinck. Pre- and reconditioning of DCD donor lungs on ex-vivo lung perfusion by methylprednisolone. J Heart Lung Transplant 2016;35:S143
Presented at the ISHLT, 35th Annual Meeting, April 2016, Washington (USA)

A. Martens, K. Degezelle, B. Desschans, D.E. Van Raemdonck, B.M. Vanaudenaerde, G.M. Verleden, R. Vos, S.E. Verleden, A.P. Neyrinck. How big is the pool of rejected donor lungs that potentially could become transplantable with EVLP? A single center analysis of registered donor data. J Heart Lung Transplant 2016;35:S369
Presented at the ISHLT, 35th Annual Meeting, April 2016, Washington (USA)

A. Martens, S. Ordies, B.M. Vanaudenaerde, S.E. Verleden, R. Vos, D.E. Van Raemdonck, G.M. Verleden, V. Roobrouck, J. Pinxteren, A.P. Neyrinck. Anti-inflammatory effect of multipotent adult progenitor cell administration during ex-vivo lung perfusion. Transpl Int. 2016 Oct;29(S5):5–6
Presented at the 2nd ECTTA meeting, October 2016, Barcelona (Spain)

D. Ruttens, S.E. Verleden, E. Vandermeulen, H. Bellon, J. Somers, **A. Martens**, A.P. Neyrinck, L. Dupont, B. Vanaudenaerde, R. Vos, D.E. Van Raemdonck, G.M. Verleden. Long-term outcome after lung transplantation is comparable between brain-dead and cardiac-dead donors. J Heart Lung Transplant 2015;34(4)
Presented at the ISHLT, 34th Annual Meeting, April 2015, Nice (France)

S.E. Verleden, **A. Martens**, T. Heigl, H Bellon, E. Vandermeulen, R. Vos, G.M. Verleden, J. Verschakelen, W. Coudyzer, D.E. Van Raemdonck, A.P. Neyrinck, B. Vanaudenaerde. A post-hoc analysis of donor lungs declined for transplantation. Eur Respir J 2016; 48(60)
Presented at the ERS, 26th Annual Meeting, September 2015, London (UK)

S. Ordies, **A. Martens**, S.E. Verleden, M. Boada, R. Vos, G.M. Verleden, J. Verschakelen, W. Coudyzer, D.E. Van Raemdonck, B.M. Vanaudenaerde, A.P. Neyrinck. CT-imaging of rejected human donor lungs before and after ex vivo lung perfusion. Transpl Int 2016 Oct; 29(S5):7
Presented at the 2nd ECTTA meeting, October 2016, Barcelona (Spain)

C) Abstracts presented at national conferences

A. Martens, M. Montoli, G. Faggi, J. Somers, S.E. Verleden, R. Vos, B.M. Vanaudenaerde, D.E. Van Raemdonck, A.P. Neyrinck. Validation of physiological variables during prolonged EVLP in a porcine model comparing injured versus non-injured donor lungs.
Presented at the Annual Meeting of the Belgian Transplantation Society, March 2015, Brussels (Belgium)

A. Martens, D.E. Van Raemdonck, B.M. Vanaudenaerde, S.E. Verleden, G.M. Verleden, R. Vos, A.P. Neyrinck. Pre- and reconditioning of DCD donor lungs on ex-vivo lung perfusion by methylprednisolone.
Presented at the Annual Meeting of the Belgian Transplantation Society, March 2016, Brussels (Belgium)

A. Martens, K. Degezelle, B. Desschans, D.E. Van Raemdonck, B.M. Vanaudenaerde, G.M. Verleden, R. Vos, S.E. Verleden, A.P. Neyrinck. How big is the pool of rejected donor lungs that potentially could become transplantable with EVLP? A single center analysis of registered donor data.
Presented at the Annual Meeting of the Belgian Transplantation Society, March 2016, Brussels (Belgium)

D) Awards

One of three best free oral papers presented as an abstract at the Annual Meeting of the Belgian Transplantation Society, March 2016, Brussels (Belgium). *Pre- and reconditioning of DCD donor lungs on ex-vivo lung perfusion by methylprednisolone.*

E-factor 1000 Grant – Lung Category by ECTTA Scientific Committee. Presented as an abstract at the 2nd ECTTA meeting, October 2016, Barcelona (Spain). *Anti-inflammatory effect of multipotent adult progenitor cell administration during ex-vivo lung perfusion.*

ACTA BIOMEDICA LOVANIENSIA

665. T. VANUYTSEL, Defending the Gastrointestinal Border: The Role of Impaired Intestinal Barrier Function in the Pathophysiology of Functional Gastrointestinal Disorders. 2014
666. M. NORDGREN, Peroxisome Degradation in Mammalian Cells: What pulls the Trigger? 2014
667. H. LI, The Role of Novel Antiangiogenic and Other Targeted Treatments in Soft Tissue Sarcoma. 2014
668. G. LUYEYE MVILA, Breast Cancer in the Democratic Republic of Congo: Awareness, Diagnosis and Treatment in a Country with Limited Resources. 2014
669. K. LOWETTE, The Hungry 'Little Brain': Effects of Stress and Nutrition on Neuronal Signaling in the Gut. 2014
670. C. VANORMELINGEN, Role of Inflammation and Oxidative Stress in the Pathogenesis of Gastrointestinal Dysfunction. 2015
671. Z. LI, Mechanisms of Action of Anti-TNF Therapy and Prediction of Clinical Outcomes in Inflammatory Bowel Diseases. 2015
672. V. DUBOIS, Androgen Action on Skeletal Muscle and Glucose Homeostasis. 2015
673. L. AERTS, The Regulation of PINK1 Kinase Activity: Implications for Parkinson's Disease. 2015
674. H. ZIVARI ADAB, Neural Mechanisms of Perceptual Learning in Macaque Ventral Visual Areas V4 and PIT. 2015
675. S. MEZZAR, Characterization of Hacl1$^{-/-}$ Mice, a New Animal Model for α-Oxidation Deficiency. 2015
676. L. HAKIM, Micro-Ultrasound Assessment of the Urethra in the Rat: Functional Evaluation of an Animal Model for Stress Urinary Incontinence and the Effect of Cell-Based Therapy. 2015
677. A. S. L. BARAO, BACE1 Physiological Function and its Relevance for Alzheimer's Disease Therapy and Diagnosis. 2015
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